Investigating the effect of soluble BiP on human regulatory T cell frequency and function

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Investigating the effect of soluble BiP on human regulatory T cell frequency and function

By

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Abstract

Binding Immunoglobulin Protein (BiP) is a member of the HSP70 family and is currently being used in clinical trials to treat rheumatoid arthritis. Soluble BiP has been shown to have immunoregulatory properties in murine models of arthritis and human immune cells *in vitro*. Regulatory T cells are a subpopulation of T cells which modulate the immune system, maintain tolerance to self-antigens, and abrogate autoimmune disease. Published data suggest that HSP60 and HSP70 can enhance regulatory T cell function, and therefore the ability of soluble BiP ability to affect regulatory T cell frequency and function was examined. Using the Whitehall II cohort, the concentration of soluble BiP does not correlate with regulatory T cell frequencies. Two hours of BiP pre-treatment did not enhance regulatory T cell function as demonstrated in *in vitro* suppression assays. However, treatment of responder T cells with BiP for 4 days following stimulation with anti-CD3/CD28 beads or indirectly following co-culture with monocytes treated with BiP reduced their proliferation. Since the BiP effect on responder T cells can be observed after 4 days in culture, regulatory T cells were cultured with BiP for 4 days. BiP pre-treatment for 4 days of regulatory T cells had no effect on the phenotype, cytokine secretion and function of regulatory T cells. Then, the effect of BiP on responder T cells was investigated. Responder T cells cultured with BiP revealed a significant increase in Foxp3+CD25+ cells and IL-10 secretion within the responder T cell population cultured with BiP. The BiP-induced $CD25^{High}$Foxp3+ T cell ability to suppress responder T cells were variable but these cells can reduce TNF-α secretion from autologous responder T cells in the co-culture. In conclusion, BiP may modulate responder T cell phenotype and function.

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Declaration

I declare that I have personally prepared this report and that the work described is my own unless otherwise stated. All sources of information are acknowledged by means of references.

Ahmad Mahfuz Gazali

Table of Contents

Table of Figures

Table of Tables

Abbreviations

°C	Degree celcius
1MT	1-methyl-trytophan
AA	Adjuvant arthritis
AF647	Alexa Fluor 647
AMP	Adenosine monophosphate
APC	Antigen presenting cells
ATF6	Activating transcription factor 6
ATP	Adenosine triphosphate
BBR	Berberine
BD	Beckton Dickinson
BiP	Binding Immunoglobulin Protein
BMI	Body Mass Index
C.albicans	Candida albicans
C.neoformans	*Cyrptococcus neoformans*
Ca^{2+}	Calcium
CAC	Coronary artery calcification
CD	Cluster of differentiation
CD	Crohn's disease
CD62L	Cluster of Differentiation 62 Ligand
CFA	Complete Freund Adjuvant
CHOP	C/EBP homology protein
CIA	Collagen-induced arthritis
CII	Type II Collagen
CO_2	Carbon dioxide

Conc.	Concentration
CPM	Counts per minute
CT	Computerised Tomography
CTLA-4	Cytotoxic T lymphocyte antigen 4
CVD	Cardiovascular disease
DAS 28	Disease Activity Score in 28 joints
DC	Dendritic cells
DDA	Dioctadecyl ammodium bromide
DEX	Dexamethasone
Dlgh1	Disc large homolog 1
DNA	Deoxyribonucleic acid
DSCT	Dual-source computed tomography
E.coli	*Escherichia coli*
EGFR	Epidermal growth factor receptor
eIF2α	Eukaryotic initiation factor 2 alpha
ELISA	Enzyme-linked immunosorbent assay
ER	Endoplasmic reticulum
ERK	Extracellular-signal-regulated kinases
FBS	Foetal Bovine Serum
FiTC	Fluorescein isothiocyanate
FMO	Fluorescence Minus One
Foxp3	Forkhead box3
GADD34	Growth arrest and DNA damage-inducible protein
GBO	Greiner Bio-One
GFP	Green fluorescence protein

GITR	Glucocorticoid-induced TNF receptor
GMP	Good Manufacturing Practice
GRP	Glucose Regulated Protein
H_2SO_4	Sulphuric Acid
HAS	Human Serum Albumin
HBA1c	Glycated haemoglobin
HCl	Hydrochloric Acid
HDL	High Density Lipoprotein
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HLA-DR	Human leukocyte antigen-DR
HSP	Heat Shock Proteins
IBD	Inflammatory bowel diseases
IDO	Indoleamine 2,3-dioxygenase
IFA	Incomplete Freund Adjuvant
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iNKT	Invariant Natural Killer T
IPEX	Immundysregulation, polyendocrinopathy, enteropathy, X linked
IRE1	Inositol requiring enzyme 1
IS	Immunological synapse
ITIM	Immunoreceptor tyrosine-based inhibitory
JNK	c-Jun N-terminal kinases
KDEL	Lysine-aspartate-glutamate-leucine
L	Litre

LAG3	Lymphocyte Activation Gene 3
LDL	Low density lipoprotein
LILRB1	Leukocyte immunoglobulin-like receptor subfamily B member 1
LN	Natural log
MAPK	Mitogen-activated protein kinases
MFI	Mean Fluorescence Intensity
mL	millilitre
MR	Mannose receptor
MRI	Magnetic Resonance Imaging
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide
NaOH	Sodium hydroxide
NBD	Nucleotide binding domain
NF-κB	Nuclear factor kappa beta
ng/ml	nanogram per millilitre
NLRP3	NOD-like receptor family, pyrin domain containing protein 3
NOD	Non-obese diabetic
Nrp-1	Neuropilin 1
Ns	Non significant
OD	Optical density
OIJD	Other inflammatory joint diseases
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cells

PE	Phycoerythrin
PERK	Protein kinase RNA- like endoplasmic reticulum kinase
Pg/ml	pictogram per mililitre
PKB	Protein kinase B
PKC-θ	Protein kinase C- theta
PMA	Phorbol Myristate Acetate
PP1	Protein-phosphatase 1
RA	Rheumatoid arthritis
RAG	Recombination activation gene
RAMP	Resolution associated molecular patterns
RASM	Rheumatoid arthritis synovial membrane
Reg	Regulatory
Res	Responder
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SBD	Substrate binding domain
SCID	Severe combined immunodeficient
sCTLA-4	Soluble cytotoxic T lymphocytes antigen 4
SOCS3	Suppressor of cytokine signalling 3
STAT	Signal transduction and transcription
sTNFRII	Soluble TNF-α receptor II
T1DM	Type 1 Diabetes Mellitus
TCR	T cell receptor

TF	Tissue factor
TGF-β	Transforming growth factor β
TIGIT	T cell immunoglobulin and ITIM domain
TLR4	Toll-like receptor 4
TMB	3,3',5,5'-Tetramethylbenzidine
TNFRII	TNF receptor II
TNF-α	Tumour necrosis factor alpha
TUDCA	Tauroursodeoxycholic acid
U/ml	Unit per millilitre
UC	Ulcerative colitis
UPR	Unfolded protein response
XBP1	X box-binding protein 1
µCi/ml	micro Curie per mililitre
µg/ml	microgram per millilitre
µL	microliter

Chapter 1

Introduction

1 Introduction

1.1 Heat shock protein (HSP)

Heat shock proteins were initially identified in the 1960s. HSPs or stress proteins can be defined as "proteins whose expression is increased by conditions that cause protein denaturation or unfolding in the cells" (Udono, 2012). Various conditions have been suggested to induce denaturation or unfolding of the proteins in the cells including infection, heat stress, cytokines, oxidative stress and depletion of intracellular calcium (Ca^{2+}) stores. HSP was first discovered and reported by Ritossa (1962) in the larvae of *Drosophila*. A puffing pattern was observed in the chromosomes of these larvae when the temperature of the incubator was shifted to a higher temperature. This pattern was later recognised as heat shock protein and the name was given because it was initially induced by an elevated temperature. Since then, more HSP were discovered and there are now more than 100 individual HSP identified. Since this identification, these proteins are classified into various groups according to their molecular weights. Currently, there are 6 families of HSP classified according to their molecular weights namely small HSP family, HSP40 family, HSP60 family, HSP70 family, HSP90 family and HSP110 family as reviewed by Kampinga and colleagues (2009). One of the most studied HSP as reported in the literature is HSP70, as reported by Brocchieri et al (2008). However, the laboratory has been studying Binding Immunoglobulin Protein (BiP), a member of HSP70 family and that is the focus of the project.

1.2 Binding Immunoglobulin Protein (BiP)

BiP was first discovered by Pouyssegur and colleagues (1977) when they induced BiP expression in normal chicken fibroblasts. BiP induction was observed when the cells were starved of glucose for 24-48 hours, and this protein was named as Glucose Regulated Protein 78 (GRP78). However, this protein was known as Binding Immunoglobulin Protein (BiP) after Haas and Wabl (1983) discovered that this 78kDa protein can bind non-covalently to immunoglobulin heavy chain in B lymphocyte lineage cells. Further work by Munro and Pelham (1986) confirmed that both proteins were the same protein. Therefore, this 78kDa protein can be identified by using either GPR78 or BiP. Recently, Kampinga and co-workers (2009) proposed a new nomenclature of HSP and this protein can be identified as HSPA5. However, throughout this document, this protein will be referred to as BiP.

1.2.1 BiP protein and gene

BiP belongs to HSP70 family based on its molecular weight. In addition to having an approximately similar molecular weight, BiP and HSP70 display very high homology between these two proteins (Munro and Pelham, 1986) and Daugaard and colleagues (2007) reported that human HSP70 and BiP share 64% of their amino acid sequences. Furthermore, BiP protein is a highly conserved protein structure as HSP70 and shares most of the domains with HSP70. Human HSP70 protein is 641 amino acids long and has adenosine triphosphate (ATP) binding domain, peptide binding domain, and variable region while human BiP protein (654 amino acids) displays two additional domains, namely the localisation signal at the end of N terminus and the lysine-aspartate-glutamate-leucine (KDEL) retention signal at the end of C terminus (Daugaard et al, 2007). The details of BiP structure can be seen in Figure 1.1. Panel (A) shows the linear structure of BiP where the N-terminus is flanked by signal sequence and ATPase. The N-terminus is where ATP binds to BiP. Then, the N-terminus and C-terminus is connected by a hydrophobic flexible linker before a substrate binding domain between amino acid at position 400 until 500 while at the end of C-terminus, a KDEL sequence is present. Panel (B) demonstrates the schematic diagram of BiP in its real form. However, these two proteins were encoded by two different chromosomes as HSP70 is encoded by Chromosome 7 while BiP is encoded by Chromosome 9 (Daugaard et al, 2007).

A single BiP gene is encoded in Chromosome 9. Since this protein is highly conserved among various species, a high homology of protein sequence was reported between human BiP and BiP from other species, especially mammals. For instance, human BiP shares 97% of homology compared to rat BiP as reported by Corrigall et al (2001). Furthermore, Ting and Lee (1988) reported the homology between BiP from different species. BiP genes from humans, rats, and hamsters shared unusual homology especially in the protein-coding and 3’ untranslated regions. The high degree of homology between human and rodent BiP in terms of protein sequence and gene validates the use of rats, hamsters or mice in studying this protein.

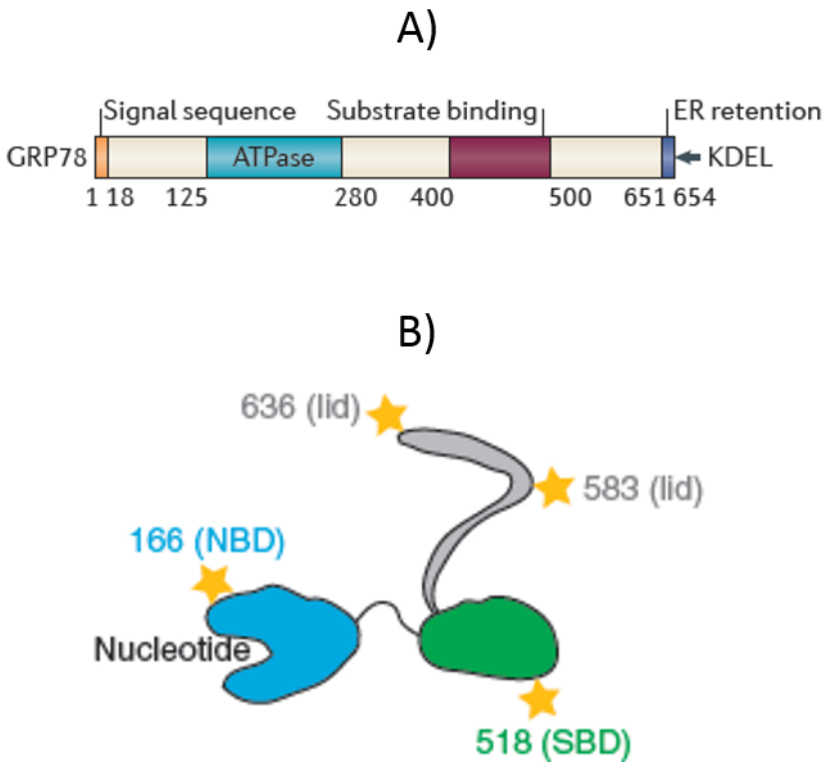


Figure 1.1 BiP protein structure. Panel A is reproduced from Lee (2014) and shows the linear representative of BiP protein. Panel B from Marcinowski et al (2011) is a schematic diagram of BiP.

NBD – nucleotide binding domain

SBD – substrate binding domain

1.2.2 The role of BiP as molecular chaperone

Molecular chaperones are proteins that assist the non-covalent folding or unfolding and the assembly or disassembly of other macromolecular structures. One of the earliest evidence of the involvement of HSP in unfolded protein response (UPR) was demonstrated in *Escherichia coli (E.coli)* by Sternberg (1973). His work showed that GroEL was essential for phage morphogenesis. GroEL only exists in prokaryotes and the homolog of GroEL in eukaryotes is HSP60 as demonstrated by more than 70% sequence homology between GroEL and human HSP60 (Bachmaier et al, 1999). The role of human HSP60 as a molecular chaperone has been well established in the literature as reviewed by Fink (1999). In addition, other HSP family members can act as molecular chaperones including BiP.

BiP was initially located in endoplasmic reticulum (ER), a cell organelle that forms from the network of membranes consisting of rough and smooth ER, and serves may functions including protein synthesis and protein transport from ER to Golgi. Proteins with correct confirmation will be transported into the Golgi. Incorrectly folded proteins induced by ER stress will undergo repair and this response is referred to as the UPR (Malhotra and Kaufman, 2007). In addition, UPR aims to degrade misfolded proteins and activate the signalling pathways that lead to increased production of molecular chaperones involved in protein folding. If these objectives are not achieved within a certain time lapse or the disruption is prolonged, the UPR aims towards apoptosis. In its inactive form, BiP binds to all three signalling branches, namely Protein kinase RNA- like endoplasmic reticulum kinase (PERK), inositol requiring enzyme 1 (IRE1) and activating transcription factor 6 (ATF6). However, when ER stress occurs, misfolded proteins bind to BiP to undergo repair and BiP will be induced to bind with misfolded proteins. As reviewed by Lee (2014), ER stress can be triggered by both intrinsic and extrinsic factors such as altered cell metabolism, hyperproliferation, hypoglycaemia, hypoxia, acidosis, viral infection and genetic lesions. All these conditions will lead to increased BiP expression in ER to cope with an increase of unfolded proteins in ER and induce signalling pathways as shown in Figure 1.2.

Signalling pathways which can be induced after BiP binding to misfolded proteins consists of three branches; PERK, IRE1 and ATF6. PERK is a type I transmembrane protein that control protein translation into ER. This is achieved by PERK activation that inhibits the initiation factor eukaryotic initiation factor 2 alpha (eIF2α) by phosphorylation. Schroder and Kaufman (2005) showed that inhibition of eIF2α activation can help to alleviate ER stress by reducing the amounts of misfolded proteins in the ER. Another branch of ER stress signalling is through ATF6. This protein is a transmembrane protein and can be activated by proteolysis as reported by Haze et al (1999). Once BiP no longer binds to ATF6, this protein will translocate to Golgi and is proteolytically cleaved by Golgi-resident site 1 protease and site-2 protease. This process will cause the release of the transcription factor domain from the Golgi as reported by Haze et al (1999). The translocation of ATF6 from the Golgi to nucleus induced the expression of genes related with protein folding, protein secretion and protein degradation. Finally, the IRE1 signalling branch can be activated when released from the BiP proteins to achieve the aims of UPR. IRE1 is a protein kinase and endoribonuclease;

therefore it can be called as a bifunctional enzyme. Parmar and Schroder (2012) reported that IRE1 can be activated after release from BiP by homodimerisation and autophosphorylation. Active IRE1 then cleaved the mRNA encoding X box-binding protein 1 (XBP1) to induce gene expression involved in protein related processes as demonstrated by Ron and Hubbard (2008). The activation of all three signalling branches in UPR will eventually lead to the induction of ER chaperones, amino acid metabolism, protein degradation, and other genes as shown in Figure 1.2.

The role of the BiP protein is not only limited to the UPR as has been demonstrated in the literature. It has been shown to be involved in the regulation of calcium homeostasis, export and degradation of proteins, co-receptor for viral entry into cells and signalling transduction (Ni et al, 2011). Multiple biological functions of BiP reflect the localisation of BiP which now has been reported to be beyond the ER.

1.2.3 BiP localisation beyond the endoplasmic reticulum

Various reports in the literature suggest that BiP can be localised in other compartments inside a cell. Apart from its main location in the ER, BiP can be found to be located in other cell organelles such as the nucleus and mitochondria. Matsumoto and Hanawalt (2000) showed the presence of BiP in the nuclei of human fibroblasts after photoactivation treatment of these fibroblast. On the other hand, translocation of BiP to nucleus was reported in cancer cells by Huang et al (2009). They showed that human hepatoma HepG2 cells can induce BiP expression in the nucleus following an intracellular increase of reactive oxygen species (ROS) concentrations inside these cells. Ni et al (2011) hypothesised that BiP localisation in the nucleus may play a role against deoxyribonucleic acid (DNA)-damaged induce apoptosis. On the other hand, Sun et al (2006) showed that BiP can be present in the mitochondria of rat brain tumour cells. BiP localisation in the mitochondria was observed by confocal microscopy and validated by immunoelectron microscopy. They suggested that BiP localisation in the mitochondria will enable it to physically interact with GRP75 as both proteins were found to be localised in the same place.

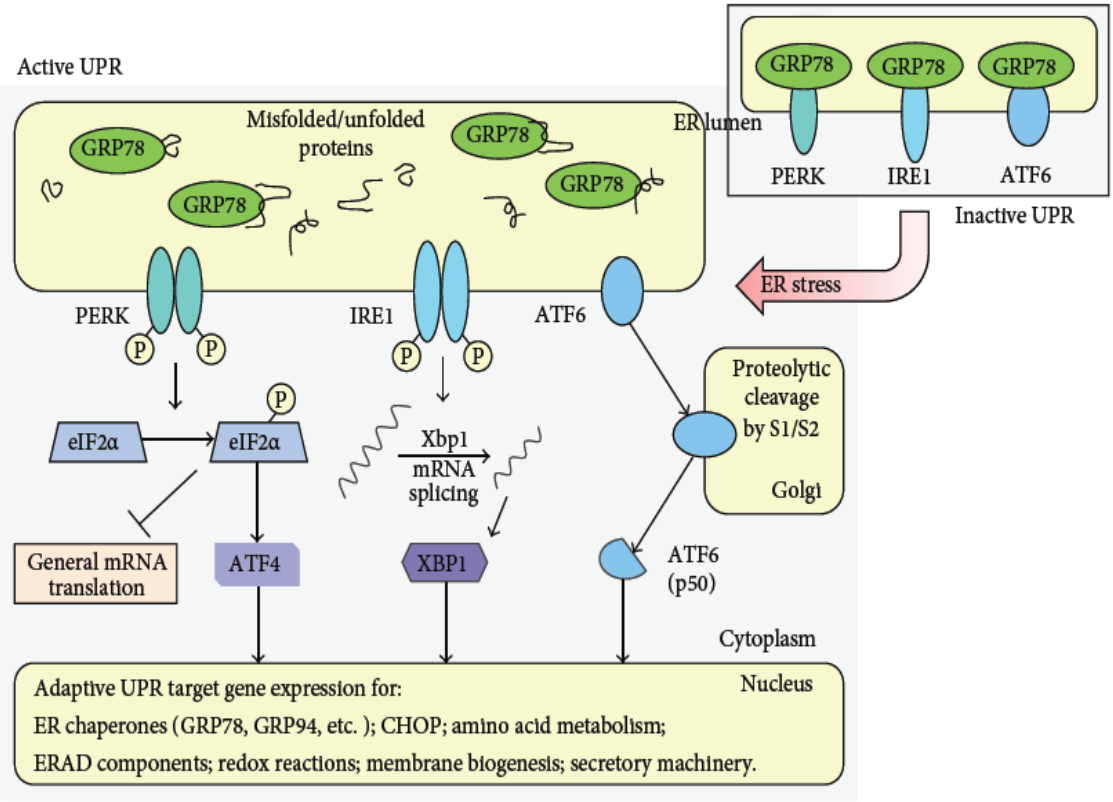


Figure 1.2. The role of BiP as a molecular chaperone. This figure is reproduced from Schonthal (2012). In normal conditions, BiP binds to PERK, IRE1 and ATF6 as shown in the right. However, when cells undergo ER stress, BiP binds to misfolded proteins and the signalling cascades were triggered via PERK, IRE1 and ATF6 signalling branch to induce UPR gene expression.

Not only can BiP be found in the cell organelles such as ER, nucleus and mitochondria, it can also be localised outside cell organelles especially on the cell surface. Berger and colleagues (1997) first reported BiP presence on the cell surface of human cutaneous T-cell lymphoma tumor cells. The protein was initially named as BE2 protein but it was later confirmed that it was homologous to BiP as both proteins can bind strongly to ATP. The BiP presence of the cell surface was confirmed in cancer cells from cell lines (Xiao et al, 1999) or freshly isolated cancer tissues (Shin et al, 2003). Most importantly, Xiao and colleagues (1999) demonstrated that BiP present on cell surfaces maintained a similar protein structure as the KDEL sequence at the C-terminus of the protein was detected. The BiP presence of the cell surface of cancer cells may represent a therapeutic target in cancer.

Furthermore, cell surface BiP was reported to be expressed by normal cells. BiP localisation on cell surfaces was observed in murine endothelial cells as reported by Bhattacharjee et al (2005). Inhibition of BiP in these cells resulted in increased procoagulant activity and they suggested that BiP negatively regulates procoagulant activity by physically interacting with the tissue factor (TF) extracellular domain on the cell surface. Cell surface BiP can be used by pathogens, especially viruses, to mediate their entry into the cells. BiP acted as a co-receptor of coxsackievirus A9 to mediate entry into the cells (Triantafilou et al, 2002) while BiP may probably functions as part of a receptor complex to mediate dengue virus entry into liver cells (Jindadamrongwech et al, 2004). Therefore, BiP localisation in different cell compartments in various cell types may represent the pleiotropic nature of this protein.

1.2.4 Active secretion of soluble BiP by different cell types

BiP localisation has been reported to be beyond its original location of ER. The presence of cell-free or extracellular BiP has been demonstrated in bodily fluids in the literature. Soluble BiP present in body fluids was shown to be slightly different in terms of protein structure. Munro and Pelham (1987) reported that the KDEL sequence at the end of C-terminus may function as a retention signal to keep BiP protein in ER. Therefore, the expression of the KDEL sequence was examined in soluble BiP. Using Western blot, Corrigall and colleagues (2004) showed that soluble BiP found in the synovial fluid was devoid of this sequence. To confirm this finding, Marin-Briggiler and co-workers (2010) investigated the structure of soluble BiP found in the oviductal fluids from women in the periovulatory period. They demonstrated that soluble BiP present in oviductal fluids was devoid of the KDEL sequence confirming that a common mechanism is used to secrete soluble BiP. Therefore, Shields et al (2011) postulated that BiP may be cleaved or alternatively spliced to generate its soluble form of protein. BiP transport to the extracellular space may be mediated by the exosomes as Gupta and Knowlton (2007) showed that HSP60 release into the extracellular space used exosomes in rat cardiomyocytes. Having reported that soluble BiP can be present in body fluids such as synovial and oviductal fluids, the presence of soluble BiP was also reported in circulation.

Soluble BiP presence in the circulation was first reported by Blass and co-workers (1995) as they showed that BiP immunoreaction was most dominant in the majority of the

sera of rheumatoid arthritis (RA) patients. Furthermore, Delpino and Castelli (2002) showed that small amounts of circulating; free BiP was detected in the sera of healthy individuals. The low concentration of extracellular BiP made it difficult for the protein to be detected. They suggested that BiP detection from healthy controls can be performed after the enrichment by ATP-agarose affinity chromatography. Furthermore, Shields and colleagues (2011) reported that BiP concentration in the sera of RA patients can be detected in the range of nanogram per millilitre. However, the mean concentration of soluble BiP was significantly lower compared to the sera of non-RA patients. The next question was whether soluble BiP is actively secreted by cells or the soluble form of this protein present in body fluids was merely due to the release of this protein from dead cells.

Using human rabdomiosarcoma cells, a type of connective tissue cancer, BiP secretion into a cell culture medium was demonstrated by Delpino and Castelli (2002). The cells were stimulated with thapsigargin to induce ER stress which subsequently leads to the secretion of extracellular BiP. To confirm that the extracellular BiP release into cell culture medium was due to active secretion of intact cells, brefeldin A, a drug which blocks the transport of ER to Golgi, was added into the culture of these cells. Rabdomiosarcoma cells stimulated with thapsigargin in the presence of brefeldin A significantly decreased BiP secretion compared to cells cultured without brefeldin A. In addition, other cancer cells were shown to induce BiP secretion. Peng et al (2013) showed that human colon cancer cell line DLD1 can induce BiP secretion to convert mesenchymal stem cells into cancer-associated fibroblasts while Kern et al (2009) demonstrated that another colon cancer cell line, HRT-18, secreted copious amounts of BiP to block the activity of the bortezomib, an anti-tumour drug. In addition, normal cells also were associated with BiP secretion. Takemoto and colleagues (1992) showed that rat pancreatic cells can cause the secretion of extracellular BiP and the secretion was not due to the release of BiP from dead cells, but active secretion by intact cells. Furthermore, Marin-Briggiler et al (2010) showed that extracellular or soluble BiP can be secreted by human oviduct epithelial cells and this protein plays an important role in the fertilisation process. This shows that soluble or extracellular BiP can be actively secreted by different cell types and can therefore be detected in bodily fluids.

1.3 BiP and the immune response

One of the most important systems in human physiology is the immune system. The immune system consists of several biological structures and processes within an organism. By having an immune system, an organism can detect a wide variety of pathogens, distinguish them from healthy tissues and fight the pathogens. BiP can be induced in various cell types in response to infection in healthy individuals as a part of the UPR response. However, ER stress and BiP induction are related to the immune system as ER stress and the immune response share key transcription molecules downstream of the signalling pathway. IRE1, one of the sensing mechanisms used in ER stress, is the downstream signalling molecule of toll-like receptor 4 (TLR-4) as reviewed by Martinon and Glimcher (2011). Therefore, cross-talk of ER stress response and immune response is possible and this section aims to examine the effect of BiP induction via ER stress in immune cells in modulating immune cell functions.

1.3.1 BiP induction can trigger immune responses

BiP induction in immune cells can be divided into innate and adaptive immune cells. Innate immune cells include mast cells, monocytes, macrophages, dendritic cells, natural killer cells and γδ T cells. Using THP-1 cells and primary human monocytes, Komura and colleagues (2013) reported that tunicamycin can induce the expression of ER stress related proteins including BiP. They showed that tunicamycin-induced-ER stress can reduce the expression of pro-inflammatory cytokines of tumour necrosis factor alpha (TNF-α) and interleukin 1 beta (IL-1β) in human monocytes in response to lipopolysaccharides (LPS) stimulation. This was due to the attenuation of nuclear factor kappa beta (NFκB) signalling in these cells which results in the reduction of the secretion of the cytokines. In addition, they showed that ER stress can impair the ability of human monocytes to differentiate into macrophages. Menu et al (2012) demonstrated that human macrophages can upregulate IL-1β when subjected under ER stress using three compounds; tunicamycin, brefeldin A and thapsigargin. The release of IL-1β from macrophages was due to the activation of the NOD-like receptor family, pyrin domain containing protein 3 (NLRP3) inflammasome. NLRP3 inflammasome is one of the pattern recognition receptors that can sense endogenous danger signals and they showed that it can be activated by ER stress. In addition, human monocyte-derived dendritic cell (DC) also reported the pro-inflammatory effect of ER stress

as shown by Goodall et al (2010). ER stress induced by tunicamycin and TLR-4 stimulation by LPS significantly enhanced the transcription and secretion of IL-23 and IL-12, both pro-inflammatory cytokines compared to LPS stimulation alone. The pro-inflammatory effect of ER stress (BiP induction) effect in DC and macrophages was in contrast to the anti-inflammatory effect reported in the earlier study in monocytes. Since human DC, macrophages and monocytes are divided into subsets, it is crucial to observe the effect of ER stress on each subsets of these cells. Kenndey and co-workers (2013) examined closely the effect of ER stress on different macrophage subsets namely M1 and M2 macrophages. This classification is based on their function as M1 macrophages are associated with the pro-inflammatory phenotype while M2 macrophages are immunomodulatory. Human macrophages derived from monocytes were polarised to become either M1 (IFN-γ/LPS) or M2 macrophages (IL-4/IL-13) with the appropriate cytokine combination and ER stress was induced by using thapsigargin and tunicamycin. The results showed that ER stress can upregulate 33 pro-inflammatory genes including TNF-α, IL-8 and IL-6 and most importantly activate inflammasome to induce IL-1β secretion compared to M2 macrophages. In addition, ER stress caused M1 macrophages to be more susceptible to apopotosis but not in M2 macrophages. Therefore, the outcome of BiP induction via ER stress depends on the subsets of each immune cell.

However, the investigation of BiP induction in adaptive immune cells was limited to general cell types namely CD8+ and CD4+ T cells. ER stress had been shown to induce pro-inflammatory response in CD8+ T cells whilst the effect was contradictory in CD4+ T cells. Chang and colleagues (2012) showed that the expression of ER stress related proteins including BiP were increased in the animal model of Crohn's disease. Furthermore, they demonstrated that intraepithelial CD8+ T cells from mice with heterozygous BiP phenotype had an impaired ability to mediate granzyme-B-dependent cytotoxicity. This indicates that ER stress promotes the development and maintenance of the pathogenic intraepithelial CD8+ T cells in these mice. In contrast, human CD4+ T cells exhibited a regulatory phenotype in response to ER stress as shown by Franco et al (2010). ER stress induced by thapsigargin caused an increase of the expression of ER stress related proteins including BiP, C/EBP homology protein (CHOP) and growth arrest and DNA damage-inducible protein (GADD34). Interestingly, ER stress induction in CD4+ T cells strongly induces IL-10 secretion and IL-10

secretion was dependent on the eIF2α as salubrinal, a small molecule inhibitor of eIF2α dephosphporylation, dramatically inhibited IL-10 secretion. In conclusion, BiP induction via ER stress can induce a variable immune response either pro or anti-inflammatory response depending on the cell types.

1.3.2 Soluble BiP can modulate immune cell functions

BiP induction can activate either pro or anti-inflammatory immune response in immune cells but evidence from literature suggests that soluble BiP can induce immunoregulatory phenotypes in immune cells. One early step in investigating the potential immunoregulatory property of any molecule is to culture the molecule with peripheral blood mononuclear cells (PBMC). Corrigall and colleagues (2004) demonstrated the potency of soluble BiP to induce IL-10 in human PBMCs. Upon culture of PBMCs with BiP, IL-10 can be significantly upregulated in the culture after 24 hours. The upregulation of IL-10 in the culture was paralleled with the downregulation of TNF-α. In addition, supernatants from PBMC culture with BiP revealed a reduction of IL-1β secretion, a pro-inflammatory cytokine associated with RA and an increase of the soluble TNF-α receptor II (sTNFRII) which act as a natural inhibitor of TNF-α. Furthermore, this work showed that soluble BiP can downregulate the expression of cluster of differentiation 86 (CD86) and human leukocyte antigen-DR (HLA-DR) in human monocytes suggesting that soluble BiP can inhibit the antigen-presenting function in monocytes as these two surface proteins were involved in activating T cells. To further investigate the effect of soluble BiP on antigen-presenting function, dendritic cells were cultured with soluble BiP. Further work by the same group (Corrigall et al, 2009) demonstrated that BiP-treated DC can induce the downregulation of CD86 and HLA-DR from monocyte-derived DC. Furthermore, these dendritic cells increase the expression of the intracellular indoleamine 2,3-dioxygenase (IDO) and leukocyte immunoglobulin-like receptor subfamily B member 1 (LILRB1) on their surface when cultured with BiP and secreted copious amounts of IL-10. However, the effect of soluble BiP is not only limited to innate immune cells.

The effect of soluble BiP on adaptive immune cells had been investigated in CD8+ and CD4+ T cells. Bodman-Smith and colleagues (2003) demonstrated the ability of soluble BiP to induce IL-10 secretion in CD8+ T cells from healthy individuals. In addition, some of the CD8+ T cell clones isolated from healthy individuals can induce the secretion of IL-4 and

IL-5, two cytokines associated with Th2 cells when cultured with soluble BiP. These clones express CD25, CD28, CD80 and CD86 molecules on the surface but not CD56 or CD57. In addition, the T cell phenotype can be modulated indirectly by BiP via BiP-treated DC as shown by Corrigall et al (2009). BiP-treated DC can induce the development of human regulatory T cells when co-cultured with T cells. These regulatory T cells expressed CD25 and CD27 molecules on their surface and had higher levels of intracellular cytotoxic T lymphocyte antigen 4 (CTLA-4) compared to T cells after culture with untreated DC, and can suppress the proliferation of autologous T cells when co-cultured with autologous responder T cells. This shows that soluble BiP has immunomodulatory properties on immune cells.

1.4 BiP and the autoimmune diseases

In addition to functioning as a molecular chaperone, BiP has been shown to play significant roles on immune cells by the induction of BiP via ER stress or the immunoregulatory properties of soluble BiP. For this reason, the role of BiP in autoimmune diseases was examined. With more than 80 diseases classified as autoimmune diseases, the top 5 autoimmune diseases were chosen based on their prevalence in the UK. Boelaert and colleagues (2010) listed the top 5 autoimmune diseases as Hashimoto's disease (hypothyroidism), Graves's disease (hyperthyroidism), rheumatoid arthritis (RA), Type 1 Diabetes Mellitus (T1DM) and finally inflammatory bowel diseases (IBD). However, after an extensive literature search, very few papers (fewer than 5 papers) were found to discuss the role of BiP directly or ER stress indirectly in hypothroidism and hyperthyroidism. Therefore, the BiP or ER stress role in autoimmune diseases will be discussed in T1DM, IBD and RA.

1.4.1 Type1 Diabetes Mellitus

Type 1 Diabetes Mellitus (T1DM) is a form of diabetes mellitus, a group of metabolic diseases in which there are high blood sugar levels over a prolonged period. It occurs when insulin-producing β cells in the pancreas have been destroyed by an autoimmune reaction hence leading to lack of insulin. The expression of ER stress related proteins was examined from the pancreatic specimen, as reported by Hopfgarten and colleagues (2014). They showed that there was no difference between the expressions of ER stress proteins, including BiP and XBP-1 between the specimen from T1DM patients and healthy controls, and the pancreatic specimen revealed an increase of ATF4, a transcription factor related to

the ER stress gene. However, the role of ER stress including BiP cannot be totally excluded in the disease as the sample number used in this data were very small (n=2). To confirm the role of BiP in T1DM, an investigation of the role of this protein and ER stress can be obtained from animal models. Oyadomari et al (2001) showed that ER stress plays a role in the destruction of β cells of the pancreas in murine pancreatic cell lines. Excessive nitric oxide (NO) production was identified to induce apopotosis to β cells and they demonstrated that ER stress can be induced by NO production marked by the upregulation of CHOP expression, a downstream signalling molecule involved in ER stress, hence leading to the death of β cells. However, in contrast to Oyadomari et al (2001), Engin et al (2013) showed that restoration of ER stress can protect mice from T1DM. The administration of ER stress mitigator tauroursodeoxycholic acid (TUDCA) at the prediabetic stage significantly reduced the incidence of T1DM in non-obese diabetic (NOD) mice. The reduction of T1DM incidence was achieved by lower cellular infiltration in the pancreas, lower apoptosis of β cells in the pancreas and most importantly restored expression of ER stress mediators including BiP. The conflicting roles shown in the literature may suggest ER stress plays a different role at the different stages of the disease but it did emphasize that ER stress may an important role in the disease itself.

1.4.2 Inflammatory Bowel Disease (IBD)

Inflammatory bowel disease (IBD) can be defined as a group of inflammatory conditions of the colon and small intestine. Best know examples of IBD are Crohn's disease (CD) and Ulcerative colitis (UC). In CD, the inflammation occurs in the lining of the digestive system, therefore it can affect any part of the digestive system from mouth to the anal canal but it commonly occurs in the last section of the small intestine (ileum) or the large intestine (colon). However, the inflammation in UC is normally limited to specific areas of the digestive system which are the colon and rectum. The involvement of BiP and ER stress was confirmed by the presence of BiP from the histology staining of the digestive system from IBD patients. Deuring and colleagues (2012) demonstrated that BiP expression was the highest in active IBD patients compared to inactive IBD patients and healthy controls from the intestinal biopsy. The presence of BiP in active IBD patients was shown in proteins and mRNA expression from the biopsies. The same finding was observed by Bogaert and co-workers (2011) as they reported higher expression of BiP proteins and messenger

ribonucleic acid (mRNA) from the biopsies of IBD patients compared to healthy individuals. Clearly this suggests that the activation of ER stress which leads to BiP induction can consequently cause intestinal inflammation.

To further confirm the role of BiP in IBD patients, evidence from the literature demonstrates that modulation of ER stress including BiP induction can lead to the reduction of the severity of inflammation in the digestive tract. Das et al (2013) showed the mechanism of glucocorticoid action in reducing inflammation in mice. Intestinal ER stress induced in Winnie mice caused misfolding of the Muc2 mucin and the glucocorticoid dexamethasone (DEX) administration can suppress ER stress and the activation of the UPR leading to the restoration of the goblet cell Muc2 production. Therefore, they suggested that glucocorticoids used in IBD treatment can ameliorate ER stress by promoting the correct folding of secreted proteins and enhancing the removal of misfolded proteins from the ER. In addition, Hao et al (2012) showed that the BiP expression in human intestinal epithelial cells can be modulated by berberine (BBR), an isoquinoline alkaloid isolated from Chinese herb *Rhizoma coptidis*. The BiP expression in these cells was enhanced significantly in the presence of IFN-γ/TNF-α and tunicamycin, and they could be dampened by BBR. This compound was shown to downregulate c-Jun N-terminal kinases (JNK) phosphorylation, the level of caspase-12 and cleaved caspase-3. Subsequently it can ameliorate pro-inflammatory cytokines and induced ER stress *in vitro*. These works show that ER stress is activated in IBD, and modulating ER stress including BiP induction may be a target of treatment for IBD in the future.

1.4.3 Rheumatoid arthritis (RA)

Rheumatoid arthritis (RA) is an autoimmune disorder that results in a chronic, systemic inflammatory disorder that may affect many tissues and organs, but principally attacks flexible (synovial) joints. This disease is more common in women than men and normally affects people from the age 40 to 70 years old. Rheumatoid arthritis can make your joints swell, feel stiff and leave you feeling generally unwell and tired. Symptoms usually vary over time, and range from mild to severe. The condition can sometimes be very painful, making movement and everyday tasks difficult. There is no cure for the disease at the moment, but it can be managed by medication, surgery, supportive treatment or

alternative treatment. However, BiP has been identified as the putative autoantigen of RA and is now being used in clinical trial to treat RA.

1.4.3.1 BiP as an autoantigen of rheumatoid arthritis

BiP plays a special role in RA compared to other immune-mediated conditions as it has been identified as a putative autoantigen in RA. Two groups independently identified the role of BiP as the putative autoantigen for RA. Corrigall and colleagues (2004) reported that soluble BiP was present in the joints of RA patients using Western blot. In addition, Blass and co-workers (2001) demonstrated BiP are overexpressed in the joints of RA patients by immunostaining compared to osteoarthritis (OA) patients. Yoo and colleagues (2012) identified the localisation of BiP in the joints by using the immunostaining technique. They showed that BiP was highly expressed in the lining layer of the hyperplastic synovium. Further work by Dong et al (2009) showed that BiP was highly expressed in plasma cells that infiltrate the synovium in RA patients compared to OA patients. In addition to the joints, BiP can also be found in the circulation. In contrast to BiP, soluble BiP concentrations in RA patients were reported to be lower in the circulation compared to control diseases as reported by Shields et al (2011). Furthermore, not only was BiP protein present in RA patients, anti-BiP antibodies and BiP-specific cells were reported in RA patients.

To confirm the role of BiP as autoantigen in RA, anti-BiP antibodies were investigated in RA patients. Corrigall and co-workers (2001) reported that the sera of 30% of RA patients reacted against BiP protein, indicating the presence of anti-BiP antibodies. Further investigation by Blass et al (2001) demonstrated that anti-BiP antibody can be detected in 63% of the sera of RA patients compared to 7% of the sera of other rheumatic diseases. In addition, anti-BiP antibodies were present only to a very low degree in the sera of the healthy population (1%). Furthermore, Corrigall and colleagues (2004) investigated the presence of anti-BiP antibody in various stages of disease in RA patients. Generally, the concentrations of anti-BiP antibodies were the highest in the sera of RA patients compared to other inflammatory joint diseases (OIJD) and normal controls. Anti-BiP antibodies were detected in 71% of patients with established disease while 61% of early RA patients were present with anti-BiP antibodies in their sera. The presence of anti-BiP antibodies in RA patients supports the contention that BiP is a putative autoantigen in RA.

Finally, the presence of BiP-specific T cells was demonstrated in RA patients. Blass and colleagues (1995) reported that T cells from 70% of RA patients produced higher proliferation after re-stimulation with BiP in *in vitro* culture and this effect was dominant in RA patients with HLA-DQ phenotype. To accurately determine the presence of BiP-specific T cells, Corrigall et al (2001) investigated the proliferative capacity of PBMC from the periphery and joints of RA patients and other inflammatory joint diseases (OIJD). PBMCs isolated from the joints of RA patients had higher stimulation index in response to BiP compared to PBMC from RA patients and cells from OIJD patients. This result was validated by showing that this effect was specific to BiP only; not to other proteins. In conclusion, BiP can be identified as a putative autoantigen in RA as demonstrated by the presence of the protein in the joints or circulation (full length or soluble form), anti-BiP antibodies in the sera and finally the presence of BiP-specific T cells.

1.4.3.2 Soluble BiP can prevent and resolve arthritis in animal models

Based on the hypothesis that BiP is an autoantigen in RA, BiP was used to try to induce arthritis in animal models. One of the famous Koch's postulates is that a microorganism isolated from disease can cause disease when introduced into a healthy organism. Based on this concept, BiP immunisation was used to investigate whether it can induce arthritis in animal models. Based on this premise, Corrigall et al (2001) tried to investigate this hypothesis by using BiP injection to induce arthritis in rodents. However, BiP injection did not induce arthritis in DBA/1 or BALB/c mice. Instead, DBA/1 mice immunised by BiP developed anti-BiP antibodies in the sera on the onset of arthritis. The presence of these antibodies suggests that immune manipulation by BiP may prevent arthritis development. To address this, they envisaged a BiP injection on the onset of arthritis in the collagen-induced arthritis (CIA) model. CIA model is induced in DBA/1 mice by intradermal injection of Type II Collagen (CII) emulsified in Complete Freund Adjuvant (CFA) at the base of the tail. After 21 days, a booster injection of Incomplete Freund Adjuvant (IFA) was given as a booster and arthritis development at each footpad was visible after 6-8 weeks. The severity of arthritis was examined by the score given to each footpad from 0 to 4 and each mouse will get a maximum of 16 scores before being sacrificed. The incidence of arthritis in these models was between 50%-80% as reported by Inglis et al (2008). Then, BiP injection was performed on the onset of arthritis in the CIA model and shown to prevent the

development of arthritis in the CIA model. This was achieved by a lower incidence of RA in these mice, intact joint architecture and reduced concentration of the pathogenic CII autoantibodies compared to control mice. However, to apply this to human disease, it is important to show that BiP injection can reduce arthritis; therefore the role of BiP in suppressing arthritis was investigated.

The first evidence that BiP injection can work in suppressing active arthritis was demonstrated in the adjuvant arthritis (AA) model. AA model is induced in Lewis rat by the injection of dimethyl dioctadecyl ammodium bromide (DDA) suspended in PBS in each footpad. After 13 days, a second injection of heat-killed *Mycobacterium tuberculosis* emulsified in IFA was given and the same scoring system was used. Corrigall et al (2001) demonstrated that pre-BiP immunisation 13 days before the induction of AA significantly reduced the mean arthritic scores in these mice compared to PBS-injected mice. In addition, further work by Brownlie et al (2006) showed the efficacy of BiP in suppressing active arthritis in the CIA model. The efficacy of BiP in treating arthritis has also been demonstrated using the adoptive transfer method. Cells from BiP-immunised mice were isolated and adoptively transferred into the CIA model and these mice showed a significant reduction of arthritis measured by the mean severity score. In addition, Shields et al (2015) demonstrated that lentiviral delivery of the BiP gene into the CIA model can resolve arthritis. A single intraperitoneal injection of lentiviral vector containing BiP was compared with the green fluorescence protein (GFP) transgene in CIA model. CIA mice that received lentiviral BiP showed reduced inflammatory cell infiltration, cartilage destruction and significantly reduced pathogenic anti-CII antibodies. It is clear now that BiP can resolve arthritis via immunisation, adoptive transfer and lentiviral delivery of BiP in the experimental arthritis model.

Evidence from literature showed that BiP modulation of arthritis in the prevention and resolution of arthritis in animal models. However, it is important to investigate whether BiP has any effect on human arthritis. Because of ethical limitation, BiP injection was not possible to be performed directly on humans without sufficient experimental evidence. Therefore, a xenogeneic model can be used to illustrate the effect of BiP on human arthritis. By using severe combined immunodeficient (SCID) mice, rheumatoid arthritis synovial membranes (RASM) from RA patients who had undergone knee joint replacement were

transplanted into these mice. RASM can be successfully transplanted into SCID mice since these mice lack functional B and T cells as they have a mutation on the SCID gene on Chromosome 16. This gene is responsible for producing an enzyme which is important in the DNA recombination mechanism. Since V(D)J recombination process does not occur, therefore the development of B and T cells were affected hence humoral and cellular immunity fail to mature. Yoshida and colleagues (2011) showed that BiP injection into RASM/SCID mice successfully reduce the severity of arthritis in these mice. The severity of arthritis was clearly visible as measured by reduced cellular infiltration and antigen presentation as measured by co-stimulatory molecules. This work gives an indication that BiP modulation may have a therapeutic effect on humans.

1.4.3.3 The immune mechanisms of BiP action in resolving arthritis in animal models

As discussed in detail in the previous subsection, BiP has the ability to suppress arthritis in experimental arthritis in mice. Brownlie et al (2006) showed that BiP has the ability to prime T cells from normal mice to become Th2 cells by secreting Th2 related cytokines, namely IL-4, IL-5 and IL-10. This was confirmed when BiP-primed cells from immunised mice increased BiP-specific proliferation and the production of IFN-γ, IL-4, IL-5 and IL-10 after re-stimulation with BiP in *in vitro* culture. Most importantly, the severity of arthritis was not significantly reduced when BiP injection was performed on IL-4 knockout mice. This result highlighted the role of IL-4 as they suggested that BiP can cure arthritis in the CIA model by inducing the development of IL-4 secreting regulatory T cells.

In contrast, Shields et al (2015) explored other possible mechanisms of BiP action in suppressing arthritis by lentiviral delivery of BiP. BiP-transfected mice produced higher levels of soluble cytotoxic T lymphocytes antigen 4 (sCTLA-4) in their sera compared to control mice. In addition, the analysis of splenocytes and lymphocytes isolated from BiP-transfected mice showed an increase of CTLA-4 positive cell frequencies compared to control mice. Finally, a co-culture of pathogenic CII cells with cells from BiP-transfected mice or control mice demonstrated an increase in IL-10 and a decrease in IL-17 from the co-culture implying that BiP may induce regulatory T cell development. The work by Yoshida et al (2011) showed the importance of IL-10 in reducing arthritis in the xenogeneic model by using neutralising antibodies towards IL-10. In addition, BiP can downregulate pro-inflammatory cytokines of TNF-α and IL-6 in the joints of these mice. Therefore, BiP is able

to modulate arthritis in animal models by reducing pro-inflammatory cytokines (TNF-α, IL-6 and IL-17), upregulating anti-inflammatory molecules (IL-4, IL-5, IL-10, sCTLA-4 and CTLA-4) and inducing regulatory T cell development.

1.5 Regulatory T cells

Regulatory T cells are a subset of T cells and central for the maintenance of immune homeostasis and central tolerance and consist of between 5-10% of total CD4+ T cells. Regulatory T cells are characterised by the presence of CD4 and high expression CD25 molecules on their surface with forkhead box3 (Foxp3) as the main transcription factor. CD4 is a glycoprotein found on the surface of immune cells and acts as a co-receptor that assists T cell receptor (TCR) communication with antigen presenting cells (APC) while CD25 is an alpha (α) chain of the IL-2 receptor. The importance of Foxp3 protein as a transcription factor of regulatory T cells was highlighted in patients with Foxp3 mutation. Chatila and colleagues (2000) first demonstrated that patients with Foxp3 mutation experienced multisystem autoimmunity which was later identified as immundysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. IPEX syndrome is characterised the presence of early onset type 1 diabetes mellitus (T1DM), severe enteropathy, eczema, anaemia, thrombocytopenia, and hypothyroidism as described by Wildin and colleagues (2002). They compared IPEX patients to scurfy mice which are characterised by over-proliferation of CD4+CD8− T lymphocytes, extensive multiorgans infiltration and elevation of numerous cytokines as described by Brunkow and co-workers (2001). Further work by Fontenot et al (2005) demonstrated that Foxp3 protein can be used to identify regulatory T cell lineage. In addition to CD4+CD25+Foxp3+ regulatory T cells, more regulatory cells were discovered in CD8, B cell or myeloid cell populations. However, the focus of this project is on CD4+CD25^{High}Foxp3+ regulatory T cells.

1.5.1 CD127 as a reliable surface marker of regulatory T cells

As mentioned before, regulatory T cells can be identified by the presence of CD4 molecules, high CD25 expression and intracellular Foxp3 expression. However, activated human CD4+ T cells can transiently express CD25 and Foxp3 as reported in several studies (Allan et al, 2007, Wang et al, 2007, McMurchy et al, 2013). Therefore, to isolate human regulatory T cells from PBMCs, an additional surface marker needs to be identified for regulatory T cells. Foxp3 protein is an intracellular protein hence staining Foxp3 with flow

cytometry antibody disables the cells to be used in the culture. Various surface markers have been reported as being a marker to truly identify human regulatory T cells. However, the surface marker discussed in this subsection is a marker related with the mechanism of suppression but purely surface markers that correlate with either Foxp3 expression or the suppressive function of regulatory T cells.

One of the most commonly described surface markers for regulatory T cells is CD127. CD127 is an α chain of the IL-7 receptor and it was first described as a marker for regulatory T cells by two independent groups in 2007. Liu and colleagues (2006) showed that CD127 downregulation in human CD4+ population indicates a suppressive phenotype and the cells were anergic, as regulatory T cells are hypoproliferative *in vitro* without IL-2 stimulation. In the meantime, Seddiki and colleagues (2006) demonstrated that Foxp3 protein expression highly correlates with $CD127^{Low}$ population within $CD4+CD25^{High}$ population. Furthermore, $CD25^{High}CD127^{Low}$ cells exist in both effector/memory and naïve phenotype by examining CD45RA/RO expression and both populations manifested suppressive activity in vitro, whereas $CD25^{High}CD127^{High}$ cells did not. In addition, Yu and colleagues (2012) performed a direct comparison between several surface markers of regulatory T cells. CD127, CD39 and CD73 were compared and contrasted with each other in terms of Foxp3 expression and suppressive capacity in the *in vitro* suppression assay. The $CD4+CD25^{High}CD127^{Low}$ population had the highest expression of Foxp3 protein and the best suppressive capacity compared to $CD4+CD25^{High}CD39+$ and $CD4+CD25^{High}CD73+$ populations. Therefore, CD127 can be used to help distinguish a pure population of regulatory T cells as regulatory T cells can be defined as $CD4+CD25^{High}CD127^{Low}$ population.

1.5.2 Thymus-derived and peripherally-induced regulatory T cells

Regulatory T cells can be divided into various subsets according to the criteria of classification but the most important criteria is the origin of differentiation. Based on this criterion, regulatory T cells can be divided into two populations, natural regulatory T cells and induced or adaptive regulatory T cells. To standardise the nomenclature of regulatory T cells, Abbas and colleagues (2013) envisaged the idea of naming different subsets of regulatory T cells based on the origin of their differentiation. Natural regulatory T cells were correctly re-named as thymus-derived regulatory T cells to reflect the fact that these cells were developed in the thymus. In contrast, peripherally-induced regulatory T cells were

used to replace induced or adaptive regulatory T cells as these cells were developed on the periphery. In addition, they also introduced '*in-vitro* induced regulatory T cells' and clearly distinguish between those regulatory T cell populations generated *in vivo* versus those generated *in vitro*.

This new nomenclature system was partly introduced because there is no consensus in the scientific community on the marker that may be used to distinguish between thymus-derived and peripherally-induced regulatory T cells especially in humans. Several markers have been proposed to distinguish these two populations based on regulatory T cell study in mice, but so far none of the markers proposed can be used in humans. For instance, Helios, a member of the Ikaros transcription factor family, was proposed as a marker to identify natural regulatory T cells in mice as reported by Thornton and colleagues (2010). They showed that Helios was 100% expressed in CD4+CD8-Foxp3+ thymocytes but the expression of Helios was only around 70% from regulatory T cells on the periphery. However, subsequent papers published about Helios expression in human regulatory T cells did not support this theory. Akimova and co-workers (2011) reported that Helios can be induced during human T cell activation and proliferation, but regresses in the same cells under resting conditions. Therefore, they suggested that Helios can be used as a marker of T cell activation and proliferation. Most importantly, Himmel and colleagues (2013) showed that Helios+ and Helios- populations existed within the human natural regulatory T cell population and these two populations had similar suppressive capacity, Foxp3 expression and surface markers such as CTLA-4 and CD39. A similar story was also reported with the neuropilin 1 (Nrp-1) expression which can be used to distinguish thymus-induced and peripherally-induced regulatory T cells in mice but not in humans. Nrp-1 is a transmembrane glycoprotein and predominantly co-receptor for another class of proteins known as semaphorins. Yadav and colleagues (2012) reported that neuropilin can be used as a marker of natural regulatory T cells based on their study in mice using a combination of novel TCR transgenic mice with a defined self-antigen specificity and conventional mouse models. However, an earlier study in humans by Milpied et al (2009) showed that human regulatory T cells did not express Nrp-1 in contrast to murine regulatory T cells. However, a small population of Foxp3+ regulatory T cells in secondary lymphoid organs expressed Nrp-1

and this protein expression was induced on peripheral blood T lymphocytes upon *in vitro* activation.

Currently there is no surface marker that can be used to distinguish between human thymus-derived and peripherally-induced regulatory T cells. Nevertheless, the classification is still valid in humans as these two populations can be easily identified based on its origin of differentiation.

In contrast to thymus-derived regulatory T cells, peripherally-induced regulatory T cells have been identified in several different subsets. One of the main factors involved in regulatory T cell induction is the presence of antigens. Long and co-workers (2011) reported that low dose of self antigen can induce antigen specific Foxp3+ regulatory T cells. Most importantly, other members of HSPs can also induce regulatory T cell development which will be discussed later. Furthermore, two more subsets of peripherally-induced regulatory T cells have been described in the literature. Zheng et al (2002) demonstrated that activated human CD4+CD25- T cells can be induced to become regulatory T cells when cultured in the presence of TGF-β cytokine. These cells are Foxp3+CD25- cells and the suppressive effect of these cells were mediated by TGF-β secretion and contact-dependent mechanism. In addition, they described that regulatory T cells can be induced in a TGF-β independent mechanism. This brings us to the final subset of peripherally-induced regulatory T cells which is TR1 regulatory T cells. These cells lack Foxp3 expression and mainly secrete IL-10 as reported by Levings et al (2005). The main cytokine involved in the induction of these cells is IL-10 as IL-10-derived from DC (Levings et al (2005)), lentiviral delivery of IL-10 (Andolfi et al (2012)) or the presence of exogenous IL-10 in naive CD4+ T cells (Levings et al (2001)) had been reported to induce these cells. Furthermore, the suppressive capacity of these cells is dependent on IL-10 as reported by Dieckmann et al (2002).

In addition, the presence of other molecules can also induce regulatory T cell development in the periphery. Kang and colleagues (2012) demonstrated that Foxp3+ regulatory T cells can be induced when CD4+ T cells were cultured with 1,25-dihyroxyvitamin D3, the active metabolite of vitamin D. These cells can suppress the proliferation of CD4+ T cells and this activity was dependent on Foxp3 expression. Enhanced Foxp3 expression was mediated by the binding of this metabolite to the nuclear VD receptor

(VDR) that binds target DNA sequences known as the VD response element (VDRE) and consequenctly enhancing Foxp3 promoter activity. In addition, the generation of peripherally-induced regulatory T cells can be facilitated by the presence of IDO enzyme expressed on DC. Chen and co-workers (2008) demonstrated that IDO presence in human plasmacytoid DC can induce regulatory T cells from CD4+CD25- T cells with potent suppressor cell function. IDO can catalyze tryptophan to become kyneurenine and kyneurenine was responsible to generate regulatory T cells. In conclusion, the presence of antigens, cytokines and other molecules may influence the development of regulatory T cells in the periphery.

1.5.3 Mechanisms of suppression of regulatory T cells

The ability of regulatory T cells to maintain homeostasis and peripheral tolerance is based on the ability to regulate other immune cell types such as CD4+ T cells, CD8+ T cells and monocytes. The mechanisms of suppression can be divided into cell-to-cell and/or soluble factors. In addition, the mechanisms of suppression used in murine regulatory T cells may be slightly different from human regulatory T cells; therefore this section aims to discuss the classical regulatory T cell suppression mechanisms observed from human regulatory T cells, namely cytotoxic T lymphocyte antigen (CTLA-4), interleukin 10 (IL-10) and transforming growth factor β (TGF-β).

1.5.3.1 Cytotoxic T Lymphocyte Antigen 4 (CTLA-4)

Cytotoxic T lymphocyte antigen 4 (CTLA-4) or CD152 is a member of the immunoglobulin superfamily and can be expressed on the surface of human T cells. The CTLA-4 expression is constitutively expressed on natural regulatory T cells as reported by Dieckmann et al (2001). Cederbom and colleagues (2000) reported that CTLA-4 on the surface of murine regulatory T cells can engage CD86 molecule on dendritic cells (DC) and downregulate the CD80 molecule. The interaction of CTLA-4 on regulatory T cells with the CD86 molecule was confirmed using human regulatory T cells from RA patients. Cribbs and colleagues (2014) demonstrated that the CTLA-4 defect from RA patients failed to induce IDO enzyme expression in RA. In addition, CTLA-4 has been reported as directly affecting responder T cell function. By engaging the CD28 molecule on human responder T cells, Birebent et al (2004) showed that human regulatory T cell suppression of responder T cells was dependent on CTLA-4 while Baecher-Allan et al (2001) demonstrated that human

regulatory T cells can suppress responder T cells independently of CTLA-4. Since the suppressive capacity of human regulatory T cells can only be evaluated using *in vitro* suppression assay, therefore is it possible that the discrepancy between these two papers lies in the different culture system used. Nevertheless, surface CTLA-4 is important in the immune system *in vivo* as CTLA-4 knockout mice developed lymphoproliferative disorders and die after 2-3 weeks after birth as reported by Khattri et al (1999).

In addition to surface CTLA-4, a newer isoform of CTLA-4 has been described as expressed in human regulatory T cells. Soluble CTLA-4 produced by alternative splicing of the CTLA-4 gene, not by cleavage of surface CTLA-4, as reported by Magistrelli et al (1999). This isoform of CTLA-4 lacks the transmembrane domain of CTLA-4 and Ward and colleagues (2013) demonstrated that resting and activated human regulatory T cells expressed sCTLA-4 and sCTLA-4 produced by regulatory T cells can suppress the proliferation of CD4+ T cells and reduce IFN-γ and IL-17 from CD4+ T cells. Clearly CTLA-4 contributes to the mechanism of suppression in human regulatory T cells by surface expression or soluble CTLA-4.

1.5.3.2 Interleukin 10 (IL-10)

IL-10 protein is an anti-inflammatory cytokine and can be secreted by a wide variety of immune cells including monocytes, macrophage, T cells and B cells. The importance of IL-10 to the immune system was highlighted by the study of IL-10 in mice. IL-10 knockout mice did not develop any form of autoimmunity but they were susceptible to developing colitis in the presence of triggering flora as reported by Kuhn et al (1993). Ito and colleagues (2008) reported that IL-10 secreted by human natural regulatory T cells can modulate DC function. IL-10 secretion by regulatory T cells can attenuate DC cell function by downregulating the expression of CD86 molecules on DC. However, the suppression of human natural regulatory T cells on responder T cell proliferation in *in vitro* suppression assay was independent of IL-10 (reviewed by McMurchy and Levings, 2012). In contrast, IL-10 is used as one of the mechanisms of suppression by adaptive or induced regulatory T cells. Dieckmann et al (2001) showed that adaptive regulatory T cells or Tr1 regulatory T cells can secrete IL-10 to suppress the proliferation of responder T cells. This evidence highlights the role of IL-10 as one of the soluble factors involved in mediating regulatory T cell suppression.

1.5.3.3 Transforming Growth Factor β (TGF-β)

Transforming growth factor β (TGF-β) is a pleiotropic cytokine and can be secreted by many cell types including immune cells. This protein exists in at least three isoforms called TGF-β1, TGF-β2 and TGF-β3 and TGF-β1 is the most commonly studied isoform of TGF-β. The role of TGF-β1 in human regulatory T cells in the suppression of responder T cells is quite controversial. Oberle et al (2007) and Annunziato et al (2002) showed that the suppression of responder T cells was partially dependent on TGF-β whilst Jonuleit et al (2001) and Dieckmann et al (2001) argued TGF-β plays no role in the suppression of responder T cells in *in vitro* suppression assay. The discrepancy could be because of the set-up of the experiments. The role of TGF-β in the development of adaptive regulatory T cells from responder T cells was well documented in human *in vitro* experiments as shown by Zheng et al (2002), Fantini et al (2004) and Wang et al (2009). In addition, TGF-β secreting regulatory T cells have been shown to modulate DC function. Esquerre and co-workers (2008) demonstrated that human regulatory T cells can inhibit T cell polarisation by modulating DC cell function.

Human regulatory T cells can exert their suppression by using membrane TGF-β in addition to TGF-β secretion. Membrane-bound TGF-β was recorded to be present in adaptive or induced regulatory T cells. Savage et al (2008) reported that M2 macrophages, an immunoregulatory macrophage, can induce the development of human CD4+CD25+Foxp3+ glucocorticoid-induced TNF receptor (GITR)+ regulatory T cells. Induced regulatory T cells by M2 macrophages express membrane-bound TGF-β1 and can suppress responder T cells using cell-to-cell contact. Furthermore, Han et al (2014) reported that human hepatocellular carcinoma-infiltrating CD4+CD69+Foxp3− regulatory T cells can suppress T cell response via membrane-bound TGF-β1. Therefore, secreted or membrane-bound TGF-β play an important role in mediating one of the mechanisms of suppression in human regulatory T cells.

1.5.4 HSP and regulatory T cells

As demonstrated by Corrigall et al (2009), soluble BiP can induce the development of regulatory T cells from responder T cells via tolerogenic DC. Reports from the literature suggest that other soluble HSP can induce the development of regulatory T cells. For instance, de Kleer and colleagues (2010) demonstrated that HSP60 can convert human

CD4+CD25- T cells to CD4+CD25+Foxp3+CD30+ regulatory T cells after being co-cultured with monocytes and HSP60 for 6 days. Furthermore, van Herwijnen and co-workers (2012) reported that HSP70, a member of the HSP70 family can induce CD4+CD25+Foxp3+ which can suppress proteoglycan-induced arthritis in mice. These works clearly demonstrate the ability of soluble HSP to induce the development of regulatory T cells.

The aim of this project is to investigate the effect of soluble BiP on regulatory T cell function. A literature search revealed that soluble HSP60 and HSP70 had been shown to modulate regulatory T cell function. Zanin-Zhorov and colleagues (2006) first described the ability of HSP to modulate human regulatory T cell function. They demonstrated that HSP60 pre-treatment on regulatory T cells can enhance their suppressive function. Regulatory T cells pre-treated with HSP60 can further suppress the proliferation of responder T cells and reduce IFN-γ and TNF-α level from the co-culture. They proposed that regulatory T cells pre-treated with HSP60 can suppress responder T cells by cell-to-cell contact and by using anti-inflammatory cytokines IL-10 and TGF-β. In addition, Wachstein et al (2012) demonstrated that HSP70 can also enhance human regulatory T cell function. HSP70 pre-treated regulatory T cells can enhance IL-10 and TGF-β secretions and reduce proliferation of responder T cells and the levels of pro-inflammatory cytokines IFN-γ and TNF-α in the co-culture. In addition, HSP70 pre-treatment on regulatory T cells can expand regulatory T cell population as measured by Ki-67 staining. Both papers demonstrated that HSP60 and HSP70 bound to TLR2 and activated the same downstream signalling molecules namely AKT and p38 mitogen-activated protein kinases (MAPK) but not ERK signalling. In addition, both papers utilised plate-bound anti-CD3 antibodies to activate responder and regulatory T cells in the co-culture. To mimic physiological activation, T cells need 2 signals to be activated; one via TCR and co-stimulation. Anti-CD3 antibody stimulation represents signal one while anti-CD28 antibody stimulation represents signal two. Therefore, co-culture of responder and regulatory T cells (pre-treated with BiP) will be performed in the absence (anti-CD3 antibody) or presence of co-stimulation (anti-CD3/CD28 beads) compared to work by Zanin-Zhorov et al (2006) and Wachstein et al (2012).

1.6 Project aims

The aims of the project are as follows:-

- To investigate the relationship between circulating BiP concentrations and regulatory T cell frequencies in healthy populations
- To examine the effect of soluble BiP on human regulatory T cell phenotype
- To determine if BiP-induced phenotypic changes may lead to enhanced suppressive function in human regulatory T cells

Chapter 2

Materials and Methods

2 Materials and Methods

2.1 Participants/Donor detail

For epidemiological studies, whole peripheral blood was obtained from the Whitehall II study. This study was initiated by Professor Michael Marmot to investigate the importance of social class, psychosocial factors and life style to CVD development among British civil servants. Participants were 600 asymptomatic men and women, aged 50-71, of white European decent and donors were selected from a broad socioeconomic status. Donors underwent a psychological stress test and dual-source computed tomography (DSCT) scanning of their coronary arteries. The data for the present study were collected at the time of a follow-up DSCT at which time blood samples were also drawn for analysis. All of the clinical/biochemical data measured from these donors were obtained from our collaborator, Prof. Andrew Steptoe from the Department of Epidemiology and Public Health, University College London (UCL). All of the data were listed in Table 3.2. Thirty (30) ml of blood were withdrawn from the Whitehall II study participants into heparinised tubes. The ethical approval of the study was obtained under the title of the psychobiology of social position REC reference 97/0356. This study was approved by the Joint University College London/ University College London Hospital Committees. However, the correlation analyses were performed on 68 out of 600 donors as regulatory T cell frequency was measured from these donors.

In contrast, cone bloods and fresh peripheral blood from healthy donors were in the functional studies in Chapter 4 and 5. Cone blood is a blood by-product of plasmapheresis containing blood cells without any plasma obtained from NHS Blood & Transplant (Collindale, UK) and less than 100 ml of fresh blood withdrawn from healthy donors were used as permitted by the ethical approval. Cone blood and fresh peripheral blood were used because of limited cell numbers obtained from peripheral blood of the Whitehall II donors.

Figure 2.1 Cone blood used in *in vitro* regulatory T cell suppression assay

2.2 Peripheral Blood Mononuclear Cells isolation

Thirty ml of heparinised blood were diluted with 1x Dulbecco's phosphate buffer salt (PBS) 1X without Calcium and Magnesium (PAA, Pasching, Austria) in 1:1 ratio. As for cone bloods, 8 mL of cone blood was diluted to 60 mL with 1x Dulbecco's PBS 1X without Calcium and Magnesium (PAA). Then, 15 mL of Lymphoprep^{TM} (Axis Shield, Dundee, Scotland) was overlaid on top of 30 mL of diluted cone blood. The tube was centrifuged at 800 *g* (Top table centrifuge - Megafuge 1.0 (Heraeus Instruments, Hanau, Germany)) for 25 minutes at room temperature (RT) without any brake. The PBMC layer is an opaque layer located between plasma and Lymphoprep as shown in Figure 2.2. The PBMC layer was harvested into cold PBS (PAA) and washed twice by centrifugation at 300 *g* (Centrifuge - Minifuge RF, Heraeus Sepatech, Hanau, Germany)) for 10 minutes at 4 degree celcius (°C) (Refrigerator-Husqvarna, Huskvarna, Sweeden)). A viability test and cell count were performed with Trypan Blue solution (Sigma, Dorset, UK) using a light microscope (Olympus, Tokyo, Japan).

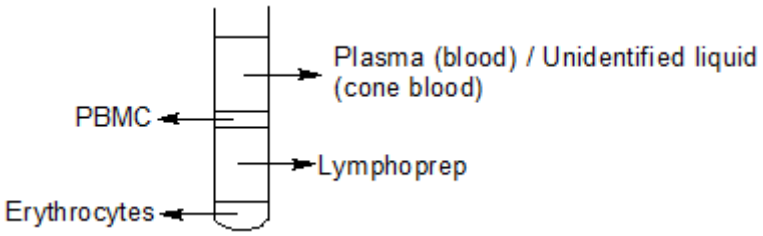


Figure 2.2. PBMC layer harvested in between plasma/unidentified liquid and Lymphoprep layer.

PBMC of donors from the Whitehall II cohort were frozen in 10% dimethyl sulfoxide (DMSO) (Sigma Aldrich, Missouri, USA) in Fetal Bovine Serum (FBS)(Gold, heat inactivated) (PAA) in a cryotube (Thermo Scientific, Waltham, USA) & placed in -80°C freezer (Triple Red Laboratory Technology, Bucks, UK) for further analysis while plasma was harvested & frozen in -20°C freezer (LEC, Bognor Regis, UK) for circulating BiP & endogenous cytokine analysis. In contrast, PBMC from cone blood and fresh blood from healthy donors were re-suspended in 1x PBS (PAA) before isolating appropriate cell populations.

2.3 Regulatory & Responder T cell isolation

Three (3) to 5 x 10^8 of PBMC from cone blood were used to isolate regulatory and responder T cells using Human CD4+CD25+ Regulatory T Cell Isolation Kit (Miltenyi Biotech, Gladbach, Germany) according to the manufacturer's instructions. Briefly, 1 x 10^7 PBMC were suspended in 90 µL of MACS buffer (see appendix) and incubated with 10 microlitre (µL) CD4+ T cell Biotin-Antibody Cocktail for 10 minutes at 4°C and subsequently with 20 µL of Anti-Biotin Microbeads (Miltenyi Biotech) for 15 minutes at 4°C. Then, cell isolation was performed using LD column (Miltenyi Biotech) and this column was fitted into MidiMACS Separator and rinsed with 2 mL of MACS buffer. The cell suspension was applied onto the column and washed with 1 mL of MACS buffer twice. CD4+ T cells were collected as flow through of LD column and centrifugated at 300 *g* for 10 minutes at 4°C.

After that, 1 x 10^7 CD4+ T cells were incubated with 10 µL CD25 Microbeads (Miltenyi Biotech). An MS column (Miltenyi Biotech) was fitted into MiniMACS Separator and rinsed with 500 µL of MACS buffer. Cell suspension was applied onto the column and washed with 500 µL of MACS buffer three times. Responder T cells, defined as CD4+CD25^{Low} population, were collected as flow through of MS column while regulatory T cells were collected by

washing away cells bound to the MS column. To achieve higher purity of regulatory T cells, a second MS column was used from PBMCs from cone blood as compared to regulatory T cells isolated from one MS column. However, isolation of regulatory T cells from fresh blood used one MS column because of limited cell numbers. Viability test & cell counts were performed & the purity of responder & regulatory T cell populations were assessed by flow cytometry using a combination of anti-CD4 (Becton Dickinson, New Jersey, USA), anti-CD25 (Miltenyi Biotech) & anti-CD127 (eBioscience, San Diego, USA) antibodies.

2.4 Monocyte, regulatory and responder T cell isolation

Three to 5 x 10^8 of PBMCs from cone blood were used to isolate regulatory and responder T cells using Human CD14 MicroBeads Isolation Kit (Miltenyi Biotech) according to the manufacturer's instructions. Briefly, 1 x 10^7 of PBMC were suspended in 80 µL of MACS buffer and incubated with 20 µL of CD14 Microbeads (Miltenyi Biotech) for 15 minutes at 4°C. Then, cell isolation was performed using an LS column (Miltenyi Biotech) and this column was fitted into MidiMACS Separator and rinsed with 3 mL of MACS buffer. The cell suspension was applied onto the column and washed with 3 mL of MACS buffer three times. CD14+ cells were collected by flushing out labelled CD14+ cells by pushing the plunger into the column with 5 mL of MACS buffer. Viability test & cell counts were performed & the purity of CD14+ cells was assessed by flow cytometry using anti-human CD14 antibody (eBioscience). After that, regulatory and responder T cell isolation were performed as described in Section 2.3. However, regulatory T cells were isolated using one MS column because of limited cell numbers.

2.5 Flow cytometry staining

2.5.1 Surface staining

2.5.1.1 Cellular staining

Typically 1 x 10^6 cells were used for staining. Regulatory and responder T cells were stained with a combination of anti-CD4 (Becton Dickinson, BD, Franklin Lakes, USA), anti-CD25 (Miltenyi Biotech) and anti-CD127 (eBioscience) antibodies in staining buffer (see appendix), while monocytes were stained with anti-CD14 antibody (eBioscience), thoroughly

mixed and incubated at 4°C for 20 minutes in the dark. After that, cell suspensions were washed with 1 mL of washing buffer (see appendix) and centrifuged at 300 *g* for 7 minutes. Then, the supernatants were discarded; cells were resuspended by vortexing (Grant Instruments, Cambridge, UK) and fixed by the addition of 100 µL of 2% paraformaldehyde (Alpha Aesar, Morecombe, UK). The data were acquired using FACS Calibur (BD) using appropriate settings. Routinely, 20,000 events in the lymphocyte gate (for regulatory and responder T cells) or the monocyte gate (for monocytes) were recorded for each sample and analysed using FlowJo 7.6.1 (TreeStar, Oregon, USA). The complete list of all FACS antibodies is listed in Table 2.1.

2.5.1.2 Whole blood staining

FACS staining was performed on the whole blood of a subset of the Whitehall II study donors. One hundred (100) µL of whole blood was stained with anti-CD4 (BD), anti-CD25 (Miltenyi Biotech) & anti-CD127 (eBioscience) antibodies, mixed thoroughly and incubated at RT for 20 minutes in the dark. After that, 1 mL of 1X Lysing solution (BD) was added to cell suspension and incubated for 15 minutes at RT. The, cell suspension was centrifuged at 300 *g* for 7 minutes. Then, the supernatants were discarded; cell suspension was resuspended using a vortex and fixed by the addition of 100 µL of 2% paraformaldehyde (Alpha Aesar) added into cell suspensions to fix the cells. Cell suspensions were acquired using FACS Calibur using appropriate settings. 20,000 events in the lymphocyte gate were recorded for each sample and analysed using FlowJo software. The data were acquired using FACS Calibur using appropriate settings. Routinely, 20,000 events in the lymphocyte gate (for regulatory and responder T cells) were recorded for each sample and analysed using FlowJo software.

Antibody (clone)	Manufacturer	Amount used (µL)	Isotype control (clone)	Manufacturer
Human anti-CD25-Phycoerythrin (PE) antibody (4E3)	Miltenyi Biotech	10 µL/test	PE-conjugated, Mouse IgG1 antibody	eBioscience
Human anti-CD127-Fluorescein isothiocyanate (FiTC) antibody (eBioRDR5)	eBioscience	2.5 µL/test	FiTC-conjugated, Mouse IgG2 antibody	eBioscience
Human anti-CD4-PerCP-Cy5.5 antibody (L200)	BD	2 µL/test	PerCP-Cy5.5 conjugated, Mouse IgG2b antibody	eBioscience
Human anti-Foxp3-Alexa-Fluor 647 (AF647) antibody (259D)	BioLegend (San Diego, USA)	3 µL/test	AF647 conjugated, Mouse IgG1 antibody	BioLegend
Human anti-CD14-AF647 antibody (61D3)	eBioscience	1 µL/test	AF 647 conjugated, Mouse IgG2 antibody	eBioscience
Human anti-CTLA-4-PE antibody (14D3)	eBioscience	2 µL/test	PE-conjugated, Mouse IgG2a antibody	eBioscience

Table 2.1. Antibodies used in FACS staining

2.5.2 Intracellular staining

Typically 1 x 10^6 PBMC were used for staining purpose. PBMCs were stained with anti-CD4 (BD), anti-CD25 (Miltenyi Biotech) and anti-CD127 (eBioscience) antibodies, thoroughly mixed and incubated at 4°C for 20 minutes in the dark. Cells were washed with washing buffer and centrifuged at 300 g for 5 minutes. One (1) mL of 1X Foxp3 Fix/Perm buffer (BioLegend) was added to the cell suspension and incubated at RT in the dark for 20 minutes. The supernatants were removed and the cell suspension was washed with staining buffer and centrifuged at 300 *g* for 5 minutes. Again, the supernatants were removed and washed with 1 ml of 1X Foxp3 Perm buffer (BioLegend) and centrifuged at 300 *g* for 5 minutes.

Cells were resuspended in 1 ml of Foxp3 Perm buffer (BioLegend) and incubated at RT in the dark for 15 minutes. The cells were centrifuged and the supernatants were discarded. Cells were incubated with anti-human Foxp3 antibody (BioLegend) at 4°C in the dark for 30 minutes. The cells were washed away with staining buffer twice before resuspending the cells in 0.5 mL of staining buffer. Cell suspensions were acquired using FACS Calibur using appropriate settings. Twenty thounds (20,000) events in the lymphocyte gate were recorded for each sample and analysed using FlowJo software.

2.5.3 Propidium iodide staining (viability assay)

Cell suspensions were alioquoted into a FACS tube and 2 µL of propidium iodide (PI) (Sigma) was added into the tube. Cell suspensions were incubated in 4°C for 10minutes before being acquired using FACS Calibur. Ten thousand (10,000) events in the Forward versus Side Scatter were recorded for each sample and analysed using FlowJo software.

2.6 Cell culture

Cells were cultured in Roswell Park Memorial Institute (RPMI) 1640 (PAA) media supplemented with 10% FBS (PAA) with 200 U/ml Penicillin/Streptomycin (Lonza, Basel, Switzerland), 5 mM Sodium Pyruvate (Lonza), 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (Lonza) and 20 mM of L-Glutamine (Lonza) in an

incubator (Research CO_2 Incubator (LEEC, Nottingham, UK)) at 37°C in an atmosphere of 5% carbon dioxide (CO_2). One million (1 x 10^6) cells were suspended in 1 mL of media in a 48-well flat bottom sterile tissue culture plate (Greiner Bio-One (GBO), Kremsmunster, Austria).

2.6.1 Two hours of HSP pre-treatment

Regulatory T cells were incubated with 1 nanogram per millilitre (ng/ml) human recombinant HSP60 protein (low endotoxin, Enzo Life Sciences, New York, USA) for 2 hours at 37°C before being washed twice with media. Various doses of human recombinant human BiP protein (Good Manufacturing Protocol (GMP) quality, free endotoxin kindly donated by Dr Valerie Corrigall, Department of Academic Rheumatology, KCL) was used as indicated in each experiment for BiP pre-treatment on regulatory T cells for 2 hours at 37°C in a 48-well flat-bottom sterile tissue culture plate (GBO) before being washed twice and re-suspended in 1 mL of media per 1 x 10^6 regulatory T cells. Cells were cultured at 37°C in an atmosphere of 5% CO_2 for 24 hours before a co-stain of anti-CD4 (BD), anti-CD25 (Miltenyi Biotech) and anti-CD127 (eBioscience) antibodies was performed as described in Section 2.5.1.1 or co-cultured with responder T cells.

2.6.2 Suppression assay or co-culture without monocytes

The stimulus was prepared first before any cells were plated into the 48-well flat-bottom sterile tissue culture plate (GBO). For plate-bound anti-CD3 (clone:OKT3, BioLegend) stimulation, 200 µL of 5 microgram per millilitre (µg/ml) of antibody suspension in 1X PBS (PAA) was layered on the bottom of the well of a 48-well plate (GBO) and incubated for 4 hours at 37°C before being washed twice with 1X PBS (PAA). Dynabeads Human T-Activator CD3/CD28 beads (Invitrogen, Carlsbad, USA) were re-suspended in excess media and centrifuged at 300 g for 10 minutes before being re-suspended in fresh media. Two hundreds and fifty thounds (2.5 x 10^5) of anti-CD3/CD28 beads (Invitrogen) were re-suspended into 10 µL of media and used in the culture at various concentrations stated in each experiment.

After that, cells were plated into the 48-well sterile tissue culture plate (GBO). Two hundreds and fifty thounds of responder T cells were added into 48-well culture plate under various conditions. Two hundreds and fifty thounds or 8.3 x 10^4 of regulatory T cells were

added into the wells for co-culture of responder T cells and regulatory T cells in a ratio of 1:1 or 1:0.3. Co-culture or suppression assay was performed for either 3 or 4 days unless stated otherwise. The final volume of culture for each well was 1 mL.

Seven hundred (700) µL of supernatants were harvested for cytokine analysis and replaced with fresh media. Five replicates of 200 µL were transferred into each well of 96-well sterile U bottom tissue culture plates (GBO) for the addition of 0.5 micro Curie per millilitre (µCi/ml) of tritiated thymidine (Perkin Elmer, Waltham, USA). Plates were incubated for 16-18 hours at 37°C with 5% CO_2 before being harvested onto filter mats (Wallac, Turku, Finland). After that, scintillation fluid (Betaplate Scint for Betaplate, Perkin Elmer) was put to the filter mats (Wallac) and harvested using a Mach III harvester 96 (TOMTEC, Orange USA). Conventional liquid procedures were used to measure the amount of tritiated thymidine (Perkin Elmer) incorporation by responder T cells with a rackbeta counter, 1450 Micro Beta Plus (Wallac). Results were presented as the mean counts per minute (CPM) from all replicates. Percentage of suppression was calculated using the following formula:-

$$\% \text{ of suppression} = 100 - \left[\frac{\text{(CPM of co-culture)}}{\text{(CPM of responder + CPM of regulatory T cells))}} \times 100 \right]$$

2.6.3 Co-culture of responder, regulatory T cells and monocytes

A 48-well flat-bottom sterile tissue culture plate (GBO) was coated with 5 µg/ml anti-CD3 antibody (BioLegend) and cell isolation was performed as described in Section 2.6.4. Each cell population (monocytes, regulatory and responder T cells) was placed into fresh media (Section 2.6). Two hour of 10 ng/ml of BiP pre-treatment was performed either on monocytes or regulatory T cells before they were washed twice. Eight thousands and three hundreds (8.3×10^4) of regulatory T cells or monocytes were added into the plate and incubated for 24 hours before responder T cells were added later and cultured for another 48 hours as indicated. After that, proliferation and cytokine measurements were determined as described in Section 2.6.2 and Section 2.7.1.1.

2.6.4 Cell culture with BiP

Two hundreds and fifty thounds of responder T cells or 1 x 10^5 of regulatory T cells were added into 48-well flat-bottom sterile tissue culture plate (GBO). Under certain conditions, 100 unit per mililtre (U/ml) of human recombinant IL-2 (Peprotech, Rocky Hill, USA) was added into the culture together with 10 ng/ml of BiP as indicated. The final volume of the culture was 1 mL.

Seven hundred µL of supernatants were harvested for cytokine analysis and replaced with fresh media. Regulatory T cells were co-stained with anti-Foxp3 (BioLegend) and anti-CD25 (Miltenyi Biotech) antibodies while responder T cells were co-stained with anti-Foxp3 (BioLegend) and anti-CTLA-4 (eBioscience) antibodies. The surface cell staining was performed as described in Section 2.5.1.1 followed by the intracellular staining protocol as described in Section 2.5.2 for responder T cell. For regulatory T cells, an intracellular staining protocol was used as described in Section 2.5.2.

2.6.5 Co-culture of autologous responder T cells with responder or regulatory T cells treated with 10 ng/ml of BiP for 4 days

Regulatory or responder T cells were cultured with 10 ng/ml BiP as indicated in the previous section. Cell suspensions were aliquoted, re-counted, washed and re-suspended into fresh media. In the meantime, autologous responder T cells were cultured in T25 culture flasks (Corning, New York, USA) for 4 days and re-counted, washed and re-suspended into fresh media.

Mock-treated or BiP-treated regulatory (8.3 x 10^4 cells) or responder T cells (2.5 x 10^5 cells) were co-cultured with 2.5 x $10^{5\ of}$ autologous responder T cells for 3 days in the presence of anti-CD3/CD28 beads (8.3 x 10^4 beads) (Invitrogen) so the ratio of autologous responder T cells and anti-CD3/CD28 beads (Invitrogen) was 5 cells to 1 bead. After that, proliferation and cytokine measurements were determined as described in Section 2.6.8.

2.7 ELISA

2.7.1 Cytokines

2.7.1.1 IFN-γ, TNF-α and IL-10 cytokines

Ninety six (96) well plates (Nunc Maxisorp, Thermo Scientific) were used for all ELISAs. Plates were washed 4 times after incubation with each antibody or enzyme-conjugate with washing buffer (see appendix). All antibodies or enzyme conjugates were used in 1 to 250 dilutions in assay buffer (eBioscience). All wells were filled with 50 µl of aliquot. The capture antibody was added into each well & incubated at 4°C overnight. Plates were blocked with assay buffer (eBioscience) for 1 hour at RT. Then, culture supernatants or plasmas were aliquoted into each well in triplicates & incubated at 4°C overnight. The standard was serially diluted in blocking buffer & blocking buffer was used as background.

Detection antibody was added & incubated for 1 hour at RT. After that, enzyme-conjugate was added and incubated for 30 minutes at RT. The final washing step was performed 6 times before 3,3',5,5'-Tetramethylbenzidine (TMB) substrate (eBioscience) was added into each well & incubated for 15 minutes at RT. The reaction of TMB substrate (eBioscience) was stopped by adding 2N sulphuric acid (see appendix) into each well. Plates were read at 450 nm absorbance with 570 nm as a reference wavelength using ELISA reader (Dynex MRX 2, Chantilly, USA). All antibodies are listed in Table 2.2. The optical reading (OD) of measurements were converted into concentrations using MasterPlex 2010 (Hitachi, Tokyo, Japan).

2.7.1.2 TGF-β1 cytokine

TGF-β1 was measured using two kits; one for measuring circulating TGF-β1 (eBioscience) and another kit for measuring TGF-β1 (Mabtech, Nacka Strand, Sweden) for the supernatants. The kit used for measuring TGF-β1 from eBioscience measured TGF-β1 after the activation step. This activation step included the addition of 2N hydrochloric acid (see appendix) into each well for 10 minutes at RT and neutralisation with 2N sodium hydrochloride (see appendix). 10 µL aliquot of acid and base was added into each well. The

pipette used to aliquot these solutions can take up a minimum of 10 µL, therefore repeated steps of aliquoting small volume of acid and base may introduce inconsistent volume of acid and base. This was not the problem with measuring circulating TGF-β1 from the plasma because of the abundance of samples but it can cause a problem with measuring TGF-β1 from culture supernatants with limited samples. The kit from Mabtech however did not require the activation step since it is measuring latent TGF-β1.

2.7.1.2.1 Circulating TGF-β1

All the steps involved in this ELISA were described in Section 2.6.12 except the neutralisation step. This step was performed before the samples were incubated. Ten (10) µL of sulphuric acid (see appendix) was added into 50 µL of plasma and incubated at room temperature for 10 minutes. After that, 10 µL of sodium hydroxide (see appendix) was added to neutralise the acid. The detail of each antibody is listed on Table 2.2.

2.7.1.2.2 Supernatant TGF-β1

Ninety six well plates (Nunc Maxisorp) were used for this ELISA. The plates were coated with 100 µL of α-TGF-β monocloncal antibody (Clone MT593, Mabtech) suspension in 1X PBS (PAA) at the concentration of 2 µg/ml overnight at 4°C. After that, the plates were washed with 1X PBS (PAA) twice before blocking the plate by adding 200 µL per well of PBS (PAA) with 0.05% Tween (Thermo Scientific) containing 0.1% BSA (30% solution, PAA) (blocking buffer). The blocking step was performed for 1 hour at RT and washed with washing buffer (see appendix) for 5 times.

Before samples can be aliqouted into each well, the standard was prepared by diluting the stock to make up a 120nM solution in 1X PBS (PAA) with 1% BSA (PAA). The stock was left at room temperature for 15 minutes and the mixture was vortexed. The serial dilution was performed with the top end concentration of standard of 100 pM (equivalent to 8000 pg/ml of latent TGF-β1). After that, 100 µL of supernatants were added into each well and incubated for 2 hours at room temperature and the washing step was repeated as before.

Next, 100 µL of 1 µg/ml of biotinylated antibody (Mabtech, clone MT517) was suspended in blocking buffer and incubated for 1 hour at RT. After washing the plate, 100 µL of streptavidin-alkaline phosphatase (Mabtech) diluted 1:1000 in the blocking buffer was added into the plates and incubated for 1 hour at RT. The final washing step was performed and 100 µL of phosphatase substrate (Sigma Aldrich) suspended in coating buffer was added into the plates and incubated overnight at 4°C. The reaction of phosphatase substrate was read after overnight incubation at a wavelength of 410 nm using the ELISA reader and the OD measurements were converted into concentrations using MasterPlex 2010.

ELISA kit	Antibody (clone)	Dilution	Buffer	Detection limit (pg/ml)
IFN-γ kit (eBioscience)	Capture antibody (NIB42)	1 in 250 dilutions	1x PBS	4-500 pg/ml
	Standard	1 in 2000 dilutions	Assay buffer	
	Detection antibody (4S.B3)	1 in 250 dilutions	Assay buffer	
TNF-α kit (eBioscience)	Capture antibody (MAB1)	1 in 250 dilutions	1x PBS	4-500 pg/ml
	Standard	1 in 2000 dilutions	Assay buffer	
	Detection antibody (MAB11)	1 in 250 dilutions	Assay buffer	
IL-10 kit (eBioscience)	Capture antibody (JES3-9D7)	1 in 250 dilutions	1x PBS	2-300 pg/ml
	Standard	3 in 10000 dilutions	Assay buffer	
	Detection antibody (JES-12G8)	1 in 250 dilutions	Assay buffer	
TGF-β kit (eBioscience)	Capture antibody (eBioTB2F)	1 in 250 dilutions	1x PBS	60-8000 pg/ml
	Standard	1 in 125 dilutions	Assay buffer	
	Detection antibody (eBio16TFB)	1 in 250 dilutions	Assay buffer	

Table 2.2. Antibodies used for ELISA assay to measure cytokine concentrations.

2.7.2 Circulating BiP

Ninety six well plates were used for all ELISAs. Plates were washed 4 times with washing buffer (see appendix) after incubation with 100 µL of each antibody or enzyme-conjugate. All antibodies were diluted in 2% BSA (PAA) in washing buffer. Zero point five (0.5) µg/ml of monoclonal mouse anti-BiP antibody (BD, clone:BD-610979) was added as a capture antibody into each well & incubated at 4°C overnight. Plates were blocked with 2% BSA (PAA) in 1X PBS (PAA) for 1 hour at RT. Then, plasmas were aliquoted into each well in triplicates & incubated at 37°C for 2 hours. The standard (Kindly donated by Dr. Corrigall, detection range: 300 – 1 µg/ml) was serially diluted in blocking buffer & blocking buffer was used as background.

Zero point five µg/ml of polyclonal rabbit anti-BiP antibody (Santa Cruz, San Diego, USA, clone: H-129) was added as a detection antibody & incubated at 37°C for 2 hours. Then, biotinylated polyclonal goat anti-rabbit IgG antibody (DAKO, Golstrup, Denmark) was added as a secondary antibody and incubated for 1 hour at 37°C. After that, streptavidin-HRP (BioLegend) enzyme-conjugate was added and incubated for 30 minutes at RT. The final washing step was performed 6 times before TMB substrate (eBioscience) was added into each well & incubated for 15 minutes at RT. The reaction of TMB substrate was stopped by adding 2N sulphuric acid into each well. Plates were read at 450 nm absorbance with 570 nm as a reference wavelength using the ELISA reader and the OD measurements were converted into concentrations using MasterPlex 2010.

2.8 Statistical analysis

The detail of the statistical analysis can be divided into two parts; correlation analysis performed in the Whitehall II study as shown in Chapter 3 and comparison between means between mock treatment and BiP-treatment in functional experiments as presented in Chapter 4 and 5.

2.8.1 Correlation analysis of the Whitehall II study

All data were checked for distribution by normality testing. The data obtained from ELISA were converted to natural log (LN) before normality test was performed using the Kolmogorov-Smirnov test. Then, correlation between data sets was performed using the appropriate test based on the distribution of the data. Data with Gaussian distribution was correlated using the Pearson correlation test while data with non-Gaussian distribution was correlated using the Spearman correlation test. An R value from both tests indicates the nature (positive or negative correlation) and strength of the correlation and correlations with $p < 0.05$ were considered statistically significant. All of the analyses were performed using SPSS Version 20 (IBM, Armonk, USA).

2.8.2 Correlation analysis for functional experiments

In Chapter 4 and 5, data obtained from the experiments were assumed to display non-Gaussian distribution as the sample number of each experiment is quite small (n=5-11). Therefore, all the statistical tests used were non-parametric tests. The Wilcoxon signed rank test was used to compare related data (data from the same pair) while the Mann-Whitney test was used to compare unrelated data. P value of less than 0.05 were considered statistically significant. All of the analyses were performed using GraphPad Prism Version 5.0 (GraphPad Software Inc., La Jolla, USA).

Chapter 3

The relationship between regulatory T cell frequency and circulating BiP concentration

3 The relationship between regulatory T cell frequency and circulating BiP concentration

3.1 Introduction

The aim of this chapter is to determine the relationship between regulatory T cell frequency and circulating BiP concentration. BiP, a member of the HSP70 family has been shown to be associated with various pathological conditions such as rheumatoid arthritis, tumour and cardiovascular disease (CVD). Intracellular BiP has been shown to play an important role in the development of CVD. Feaver et al (2008) showed that intracellular BiP upregulation in an *in vitro* model that mimics the environment of human blood vessels can be protective within atherosclerotic lesions. Hence, the role of extracellular BiP is being investigated in relation to CVD development. To study this, circulating BiP concentration was measured from plasma samples of the Whitehall II study.

Circulating regulatory T cell frequency was measured from the whole peripheral blood from the cohort. Evidence from literature indicates the importance of regulatory T cells in the protection against atherosclerosis, a condition in which an artery wall thickens as a result of the accumulation of fatty materials, which is a common cause of CVD. Several studies (Ait-Oufella et al, 2006, Herbin et al, 2012, Klingenberg et al, 2010, Mor et al, 2007 and Dietrich et al, 2012) showed that regulatory T cells were pivotal in reducing atherosclerosis in animal models of CVD. Several mechanisms have been proposed as to how regulatory T cells work to reduce atherosclerosis in animal models of CVD. Regulatory T cells can induce the differentiation of the anti-inflammatory M2 macrophage and inhibit pro-inflammatory M1 macrophage differentiation as shown by Liu et al (2011). In addition, immunization of mice with the low density lipoprotein (LDL) protein induces antigen specific regulatory T cells that can reduce atherosclerotic lesions as demonstrated by Klingenberg et al (2010) and Herbin et al (2012). Furthermore, Lin et al (2010) indicated that regulatory T cells can inhibit macrophage foam cell formation in atherosclerosis by cell-to-cell contact and soluble factors, namely IL-10 and TGF-β. Hence, the aim of this study was to investigate the relationship between circulating regulatory T cell frequency and CVD risk factors from the Whitehall II cohort.

Regulatory T cell can mediate suppression on various immune cells by cell-to-cell contact and/or soluble factors. IL-10 and TGF-β are the most commonly associated cytokines with regulatory T cell suppression. Although the role of both cytokines in *in vitro* suppression remains controversial, both cytokines have been shown to play an important role in regulatory T cell suppression in several *in vivo* experimental models. The role of both cytokines can be observed in a colitis model induced by the transfer of $CD45RB^{High}$ CD4+ T cells into Recombination activation gene (RAG-2) $^{-/-}$ mice. Asseman and co-workers (1999) highlighted the role of IL-10 secreted by regulatory T cells in the colitis model. Adoptive transfer of $CD45RB^{Low}$ regulatory T cells from wild type mice was able to inhibit colitis development in this model but not with adoptive transfer of $CD45RB^{Low}$ regulatory T cells from IL-$10^{-/-}$ mice. In addition, Powrie and colleagues (1996) demonstrated the significance of TGF-β in this model of colitis. The severity of colitis can be reduced when $CD45RB^{Low}$ regulatory T cells were adoptively transferred into these mice but the effect was reversed when anti-TGF-β antibodies was injected. These results showed the pivotal role played by both anti-inflammatory cytokines namely IL-10 and TGF-β in regulatory T cells in *in vivo* models and therefore these two cytokines will be measured and used for the association studies reported in this chapter.

Cytokines can be generally divided into pro and anti-inflammatory cytokines. In addition to measuring anti-inflammatory cytokines, pro-inflammatory cytokines were also measured in the cohort. Literature searches also focused on pro-inflammatory cytokines associated with BiP. Extracellular BiP has been shown to cause the downregulation of TNF-α and the upregulation of sTNFRII and IL-1 receptor antagonist when PBMCs were cultured in the presence of soluble BiP (Corrigall et al, 2004). In contrast, Yoo et al (2012) showed that TNF-α and IL-1β cytokines can upregulate BiP expression in human synoviocytes. Therefore, circulating TNF-α and IL-1β concentrations were measured from the Whitehall II cohort. All these cytokine measurements will be used in the correlation analysis presented in this chapter.

One of the aims of the Whitehall II study is to investigate the importance of various factors that contribute to CVD development. By measuring circulating BiP concentration, circulating regulatory T cell frequency, and circulating cytokines, the focus is to investigate associations between these measurement and clinical/biochemical parameters related to

CVD. The complete list of all clinical/biochemical parameters measured is shown in Table 3.2.

Most of the parameters are established risk factors of CVD such as anthropometric measures and blood pressure. One of the parameters measured in great detail is the lipid profile and each lipid form represents a different risk for CVD. Total cholesterol, triglyceride, High Density Lipoprotein (HDL), LDL and pericardial fat were measured. Cholesterol is an organic compound and is essential for the structural component of animal cell membranes and also serves as a precursor for the biosynthesis of steroid hormones, bile acids and vitamin D. Hypercholesterolemia or high cholesterol levels are strongly associated with CVD because these promote the development of atheroma, an accumulation and swelling in arterial walls, in the arteries thus leading to CVD. Triglycerides are blood lipids that help enable the bidirectional transference of adipose fat and blood glucose from the liver. Evidence from epidemiological studies indicates that high triglyceride levels in blood have been associated with CVD. HDL is the smallest lipoprotein particle and its main function is to transport cholesterol mostly to the liver. In contrast, LDL, the second largest lipoprotein particle, transports cholesterol into the arterial wall. Because of their respective functions, HDL is often known as good cholesterol while LDL has been labelled as bad cholesterol. All lipid profiles can be directly measured from the blood except pericardial fat. This refers to the local visceral fat depot with close proximity to the heart and can only be viewed via imaging techniques using Magnetic Resonance Imaging (MRI) or Computerised Tomography Scan (CT Scan). Various studies have reported strong associations of pericardial fat with other CVD risk factors such as body mass index (BMI) (Sicari et al, 2011) triglycerides (Sicari et al, 2011) and blood pressure (Rosito et al, 2008).

Another important clinical parameter measured from the cohort was coronary artery calcification (CAC). CAC was measured from the cohort because the presence of calcium in arteries was recorded in 96% of all heart attack victims. CAC measured using a CT scan can be detected years before any symptoms of cardiovascular events such as chest pain or shortness of breath appear. The last biochemical parameter measured was glycated haemoglobin (HBA1c). HBA1c measures the amount of glycated haemoglobin when a nonenzymatic reaction occurs between glucose and the N-chain of the beta chain of haemoglobin. This measurement is used to indicate blood glucose control over time and is

commonly used in diabetic patients. High value of HBA1c indicates poorer control of blood glucose which has been associated with cardiovascular complications among diabetic patients. Khaw and Wareham (2006) in their review suggested HBA1c measurement can be used as a marker of CVD risk in both diabetic patients and healthy populations. All of these clinical/biochemical measurements were provided by our collaborator, Prof. Andrew Steptoe.

All clinical/biochemical measurements will be used in correlation analysis using either the Pearson or Spearman test. The aim of this chapter is to investigate the relationship between regulatory T cell frequency and circulating BiP concentration. In this statistical analysis $p<0.05$ was regarded as significant whereas the R value indicates the strength of the association.

3.2 Objectives

Regulatory T cell frequency was measured in whole peripheral blood and acquired using flow cytometry while plasma concentration of endogenous circulating BiP and other circulating cytokines were measured in this cohort using specific ELISAs. Clinical/biochemical parameters were measured from matching subjects and the data were provided by our collaborator, Prof. Andrew Steptoe. In brief, the objectives of this chapter are as follows:-

- To measure endogenous circulating BiP concentration in plasmas from donors of the Whitehall II study using ELISA
- To measure various endogenous circulating cytokines in plasmas from donors of the Whitehall II study using ELISA
- To measure regulatory T cell frequency from whole blood in donors of the Whitehall II study using flow cytometry
- To investigate the relationship between endogenous circulating BiP concentration and regulatory T cell frequency
- To investigate the relationship between endogenous circulating BiP concentration and circulating cytokines
- To investigate the relationship between endogenous BiP concentration, immunological parameters and clinical/biochemical parameters

3.3 Results

3.3.1 Circulating BiP concentration

Investigating the immunomodulatory properties of BiP has been the focus of the lab for the past 10 years. Therefore, endogenous circulating BiP concentration was measured from plasmas of the Whitehall II study donors. As illustrated in Figure 3.1, circulating BiP levels display a wide range of concentration from 1 to 268 µg/ml.

3.3.2 Endogenous circulating cytokine concentrations

Circulating cytokine concentrations were measured in plasmas from the Whitehall II study donors. Both pro and anti-inflammatory cytokines were measured. Figure 3.2 shows levels of endogenous circulating cytokines measured in the plasma samples. All cytokines namely TNF-α, IL-1β, IL-10 and TGF-β display a wide range of concentration from undetectable up to very high levels. Figure 3.2(A) demonstrates levels of TNF-α that were detectable in 66% of donors ranging from 2 pg/ml to 1000 pg/ml ml in the 68 donors of the Whitehall II study whilst Figure 3.2(B) illustrates levels of plasma IL-1β that were detectable in 60% of samples ranging from 2 pg/ml to 35 pg/ml. The major anti-inflammatory cytokine, IL-10 was detectable in 85% of donors ranging from 2 pg/ml to 34 pg/ml in 68 donors (shown in Figure 3.2(C)) while Figure 3.2(D) portrays levels of plasma TGF-β that were detectable in 88% of donors ranging from 60 pg/ml to 1723 pg/ml.

Data in Figure 3.2(A-C) were originally generated by Dr. Frank Kaiser, a postdoctoral research associate working in the lab. To ensure the reproducibility of the data, re-measurement of plasma levels of TNF-α, IL-1β and IL-10 were performed as shown in Figure 3.3. Re-measurement of all cytokines except TGF-β showed reduction in the percentage of detection in donors. The discrepancy between these 2 measurements raises a question on which measurement should be used for the correlation study. For instance, TNF-α levels were detected in 66% of donors (Figure 3.2(A)) were reduced to only 23% on repeat measurements (Figure 3.3(A)). This pattern also can be observed on IL-1β (60% to 8%) and IL-10 levels (85% to 4%). Furthermore, the cytokine levels reduced dramatically upon re-measurement. An example can be seen in the plasma level of TNF-α of donor 417. The level of TNF-α was significantly reduced from 16 pg/ml (1st measurement) to undetectable (2nd

measurement) and similar observations can be seen in plasma levels of IL-1β (20 pg/ml to undetectable) and IL-10 (35 pg/ml to 8 pg/ml).

The initial measurements were performed by a postdoctoral research associate, Dr Frank Kaiser and the plasma samples were kept at -20 degree celcius (°C). The second measurements of cytokine levels were performed 2 years after the initial measurement. The discrepancy between these two measurements may be explained by two important variables; temperature and duration of storage. The recommended storage temperature of plasma for cytokine analysis is -70°C as suggested by Aziz et al (1999). In addition, they showed that plasma storage at temperatures of -20°C can cause cytokine degradation at faster rates compared to storage at -70°C. Furthermore, de Jager and colleagues (2009) demonstrated that plasmas even stored at -70°C can degrade after a few years of storage, especially with IL-1β which can degrade up to 30% of the baseline level after 2 years of storage. This may provide explanation on lower cytokine levels of the second measurement compared to the cytokine levels of the first measurement. Therefore, the correlation analysis of plasma cytokines will be performed using the first measurements.

3.3.3 Gating strategy to obtain circulating regulatory T cell ($CD4^{+}CD25^{High}CD127^{Low}$) frequencies from whole blood samples

Figure 3.4 shows the gating strategy used to measure regulatory T cell frequency in whole blood from 68 donors of the Whitehall II study. Each donor underwent the same regulatory T cell enumeration based on the gating strategy as shown. Regulatory T cells are defined in this study as the $CD4^{+}CD25^{High}CD127^{Low}$ population. Regulatory T cells were identified by gating the cells from (A) Lymphocyte gate followed by (B) CD4+ T cell gate & (C) CD25+CD127- population based on the CD25 against CD127 dot plot. To control for antibody staining, comparison between anti-CD4, CD25 and CD127 staining and isotype control was performed as shown in Figure 3.4 (D-F). Responder T cell ($CD4^{+}CD25^{Low}CD127^{High}$), CD4+ T cells and ratio of responder to regulatory T cells were obtained from the staining to be compared with regulatory T cell frequency.

Regulatory T cell frequency measurement was performed on the whole peripheral blood of the Whitehall II cohort. To assess the consistency hence the reliability of regulatory T cell frequency staining a number of replicate samples were stained from individual donors

Figure 3.5 demonstrates the consistency of regulatory T cell frequencies obtained in triplicate measurements as regulatory T cell frequencies obtained were 6.53%, 6.70% and 6.36% respectively. As they showed little variability single measurements were employed for the bulk of the cohort.

3.3.4 Intracellular Foxp3 staining

Human natural regulatory T cells are characterised by the presence of high Foxp3 expression, a master regulator in the development and function of regulatory T cells. Therefore, intracellular Foxp3 staining was performed to validate the gating strategy used in Figure 3.6 as regulatory T cell was defined as the $CD4^{+}CD25^{High}CD127^{Low}$ population. This procedure can only be performed on PBMC and not on whole blood. Hence, the staining procedure was performed after surface staining of PBMC with anti-CD4, CD25 & CD127 antibodies.

Figure 3.6 illustrates that 75% of the $CD4+CD25^{High}CD127^{Low}$ population are Foxp3+ cells in 3 different donors from the Whitehall II study. In comparison, responder T cells or $CD4^{+}CD25^{Low}CD127^{High}$ population consists of 12% Fopx3+ cells which confirms that human responder T cells can express low levels Foxp3. These data support the use of gating strategy validating the regulatory T cell frequency gating strategy.

3.3.5 Regulatory T cell frequency measurement

Regulatory T cell frequencies were measured in the whole blood of Whitehall II donors using flow cytometry using a combination of anti-human CD4, CD25 & CD127 antibodies. Regulatory T cell frequency as shown in Figure 3.7 displays a wide range from 2.59% to 12.9% of total CD4+ T cell.

3.3.6 The relationship between regulatory T cell frequency, BiP and circulating cytokines

Table 3.1 shows correlation analysis performed between circulating BiP and various immunological parameters. Regulatory T cell frequency negatively correlates with responder T cell frequency ($R=-0.742$, $p<0.001$) and CD4+ T cell frequency ($R=-0.998$, $p<0.001$). Therefore, any significant correlation between regulatory T cell frequency and any parameters can be cross-checked with responder T cell frequency and CD4+ T cell frequency. As mentioned before, the aim of this chapter is to investigate the relationship between regulatory T cell frequency and circulating BiP concentration. As shown in Table

3.1, regulatory T cell frequency correlation with circulating BiP concentration is statistically insignificant as demonstrated in scatter plot in Figure 3.8(A) (R=0.006,p=0.958). To confirm that circulating BiP concentration does not correlate with any cell frequency, correlation analyses were performed. Both responder T cell and CD4+ T cell frequency measurements exhibit non-significant correlations with circulating BiP concentration as demonstrated in Table 3.1 and shown in the scatter plot in Figure 3.8(B-C) (Responder T cell frequency: R=0.097,p=0.431, CD4+ T cell frequency: R=-0.008, p=0.951).

In contrast, circulating BiP concentration correlates positively with most of the cytokines except TGF-β. Circulating BiP concentration strongly correlates with circulating TNF-α as shown in scatter plot in Figure 3.9(A) (R=0.767, p<0.001). In addition, circulating BiP concentration correlates with IL-1β (R=0.271, p=0.025) (Figure 3.9(B)) and IL-10 (R=0.356, p=0.003) (Figure 3.9(C)). The scatter plot shown in Figure 3.9(D) confirmed non-significant correlation between circulating BiP concentration with TGF-β (R=0.005, p=0.969).

Correlation analyses between circulating cytokines revealed specific correlations. TNF-α correlates with IL-1β (R=0.332, p=0.006) and IL-10 (R=0.372, p=0.002) but not with TGF-β (R=-0.008, p=0.945) (not shown). Similarly, IL-1β correlates with IL-10 (R=0.352, p=0.003) but not with TGF-β (R=0.109, p=0.377) (not shown). However, TGF-β only correlates with IL-10 (R=0.268, p=0.027). These correlations were demonstrated in the scatter plots as shown in Figure 3.10.

3.3.7 Clinical/biochemical parameter measurement

The list of all clinical/biochemical parameters measured were listed in Table 3.2. These measurements were recorded and obtained from Prof. Andrew Steptoe from UCL.

3.3.8 The relationship between clinical/biochemical parameters, circulating BiP and immunological parameters

To test how robust are the data sets obtained from our collaborator, correlation analysis was performed for known associations between the various clinical parameters. For instance, it is known from the literature that weight correlates with BMI while systolic blood pressure correlates with diastolic blood pressure. This is confirmed in these data sets as shown in Figure 3.11. Weight strongly correlates with BMI as expected with R value of 0.846 (Figure 3.11(A)) while systolic blood pressure strongly correlates with diastolic blood

pressure with R value of 0.7901 (Figure 3.11(B)) (both value were p<0.0001). In addition, this shows that the analyses were performed correctly as these variables are expected to correlate with each other (weight & BMI, MSBP & MDBP). These correlations may explain a relationship between 2 variables. It can describe a linear relationship as denoted by positive R value or negative R value. Therefore, a literature search explaining the relationship between variables was performed to support these correlations.

The appropriate correlation analyses were performed between immunogical and clinical/biochemical parameters and only significant correlations (p<0.05) are shown in Table 3.3 as most of the correlations were non-significant. Correlation analyses revealed that regulatory T cell frequency does not correlate with any established CVD risk except body weight. The only correlation found between regulatory T cell frequency and clinical parameters is a negative correlation between regulatory T cell frequency and weight (R=-0.252, p=0.038) (Figure 3.12(A)), but not with BMI (R=-0.194, p=0.113) (Figure 3.12(B)). To confirm that this association is specific between regulatory T cell frequency and body weight, correlation analysis was performed between responder T cell frequency and body weight and the correlation is statistically insignificant (R=0.217, p=0.07) (Figure 3.12(C)) but CD4+ T cell frequency positively correlates with body weight (R=0.257, p=0.035) (Figure 3.12(D)).

Weak correlations can be found between immunological parameters and clinical/biochemical parameters. IL-10 correlates with body weight (R=0.256, p=0.03) and pericardial fat (R=0.264, p=0.03) as illustrated in Figure 3.13(A&C). Similar to regulatory T cell frequency, circulating IL-10 does not correlate with BMI (R=0.143, p=0.243) (Figure 3.13(B)). As for IL-10 and pericardial fat association, this is the first time such correlation was reported after extensive literature search. The only correlation between circulating BiP concentration and clinical/biochemical parameters is between circulating BiP and triglyceride level in the blood of the Whitehall II study donors. BiP negatively associates with blood triglycerides (R=-0.265, P=0.029) as shown in Figure 3.14. However, no associations were found between other blood lipids such as total cholesterol, HDL and LDL and BiP, showing that the correlations are specific to triglycerides only.

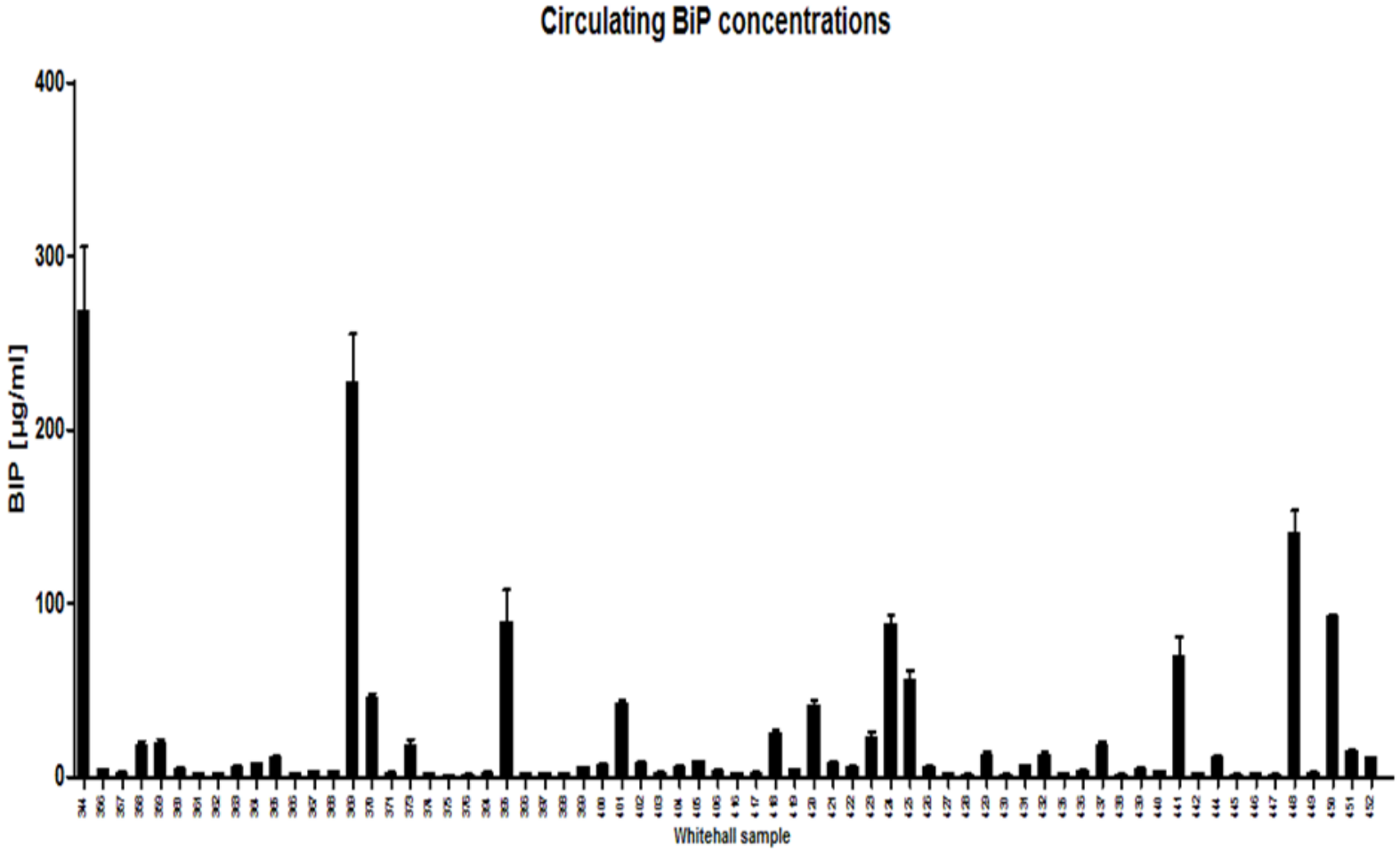
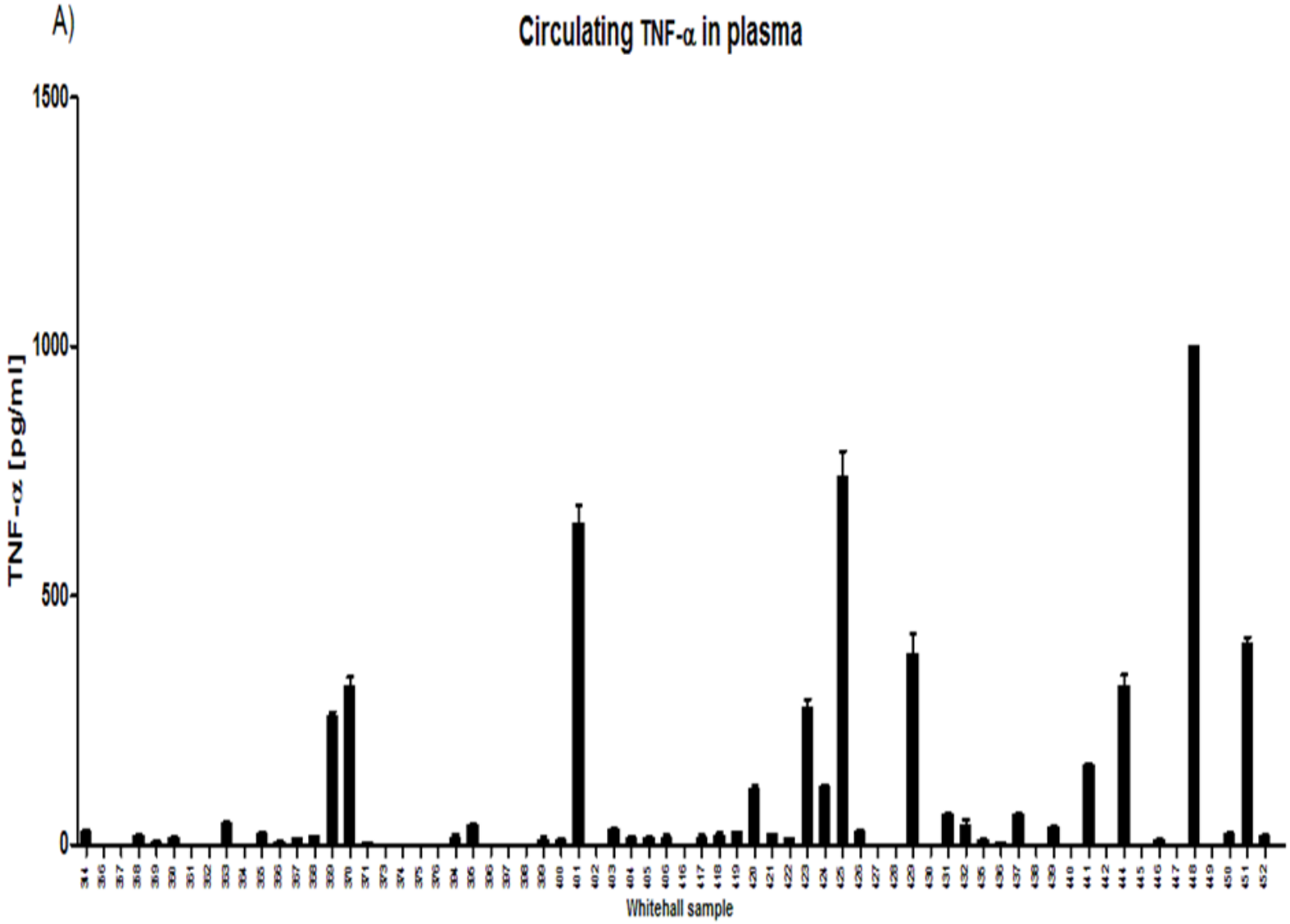
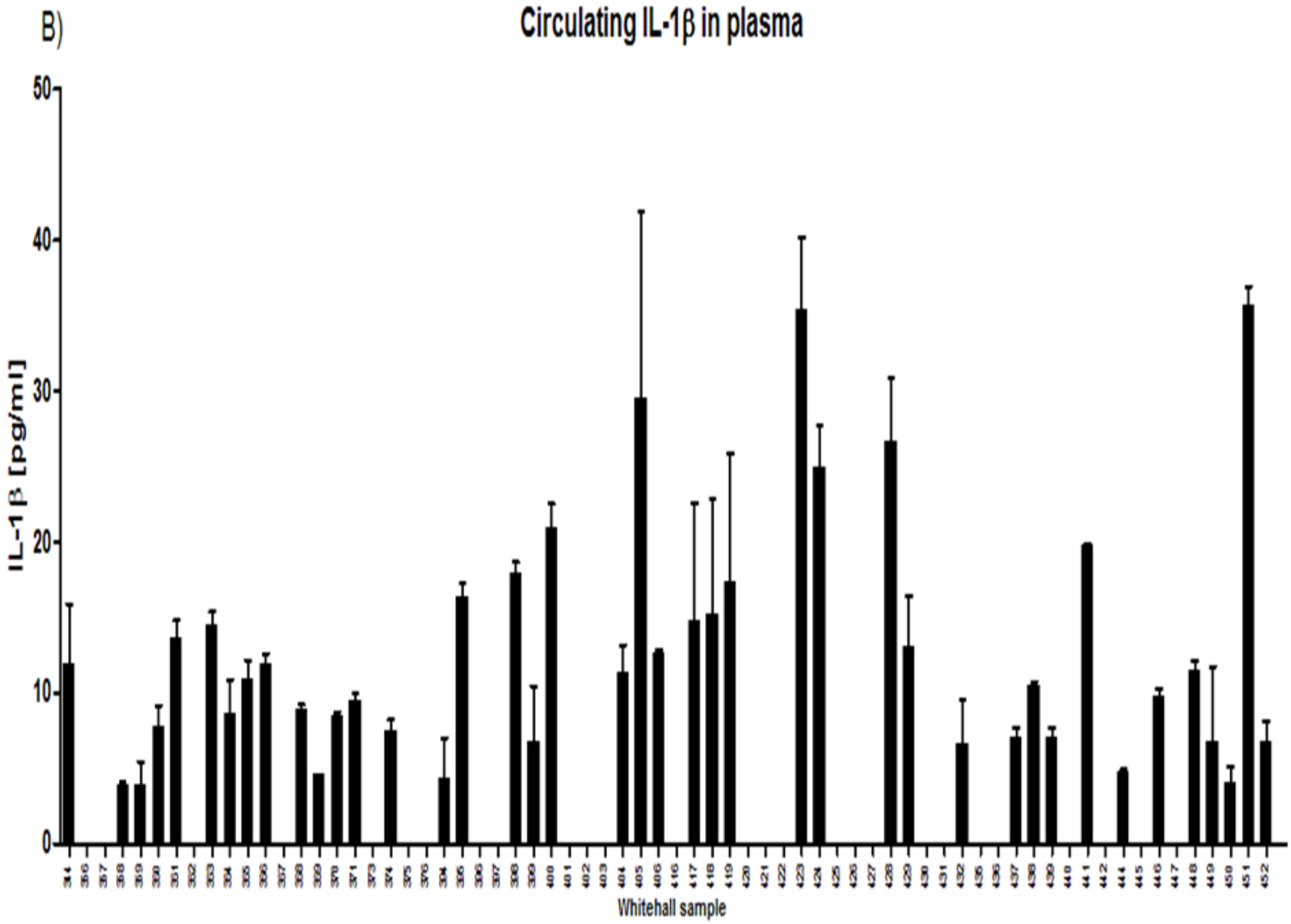


Figure 3.1. Variability of soluble BiP concentrations in plasma of the Whitehall II donors. Circulating BiP was measured using ELISA in plasma of Whitehall II donors. Each bar represents the mean of triplicate measurements of one of 68 donors and the error bar represents the standard deviation. The range of soluble BiP varies from 1 to 268 µg/ml. These data were generated by Dr Frank Kaiser, a postdoctoral research associate working in the lab.


A)
Circulating TNF-α in plasma
TNF-α [pg/ml]
1500
1000
500
0
Whitehall sample


B)
Circulating IL-1β in plasma
IL-1β [pg/ml]
50
40
30
20
10
0
Whitehall sample

C)
Circulating IL-10 in plasma
IL-10 [pg/ml]
0
10
20
30
40
Whitehall sample

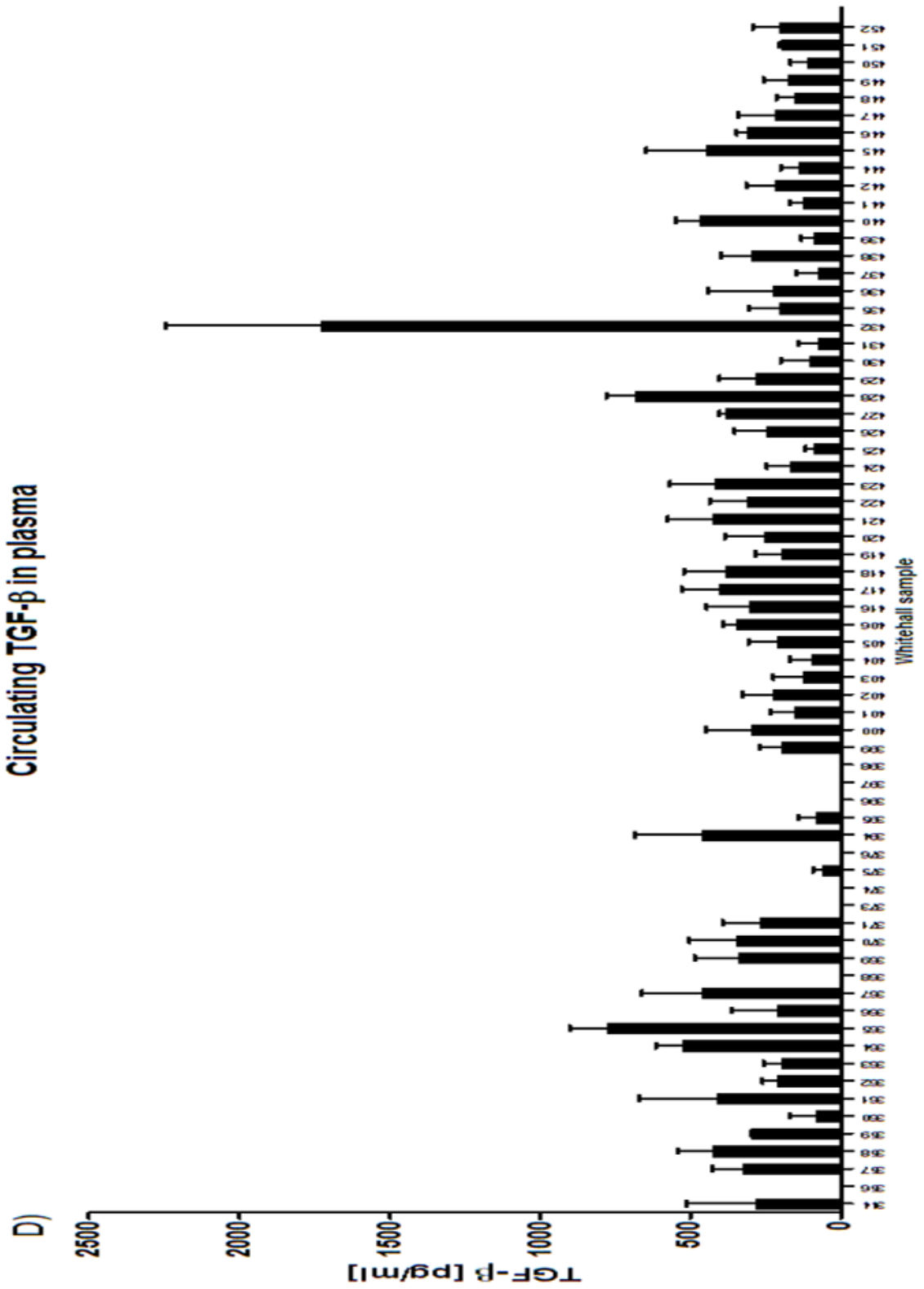
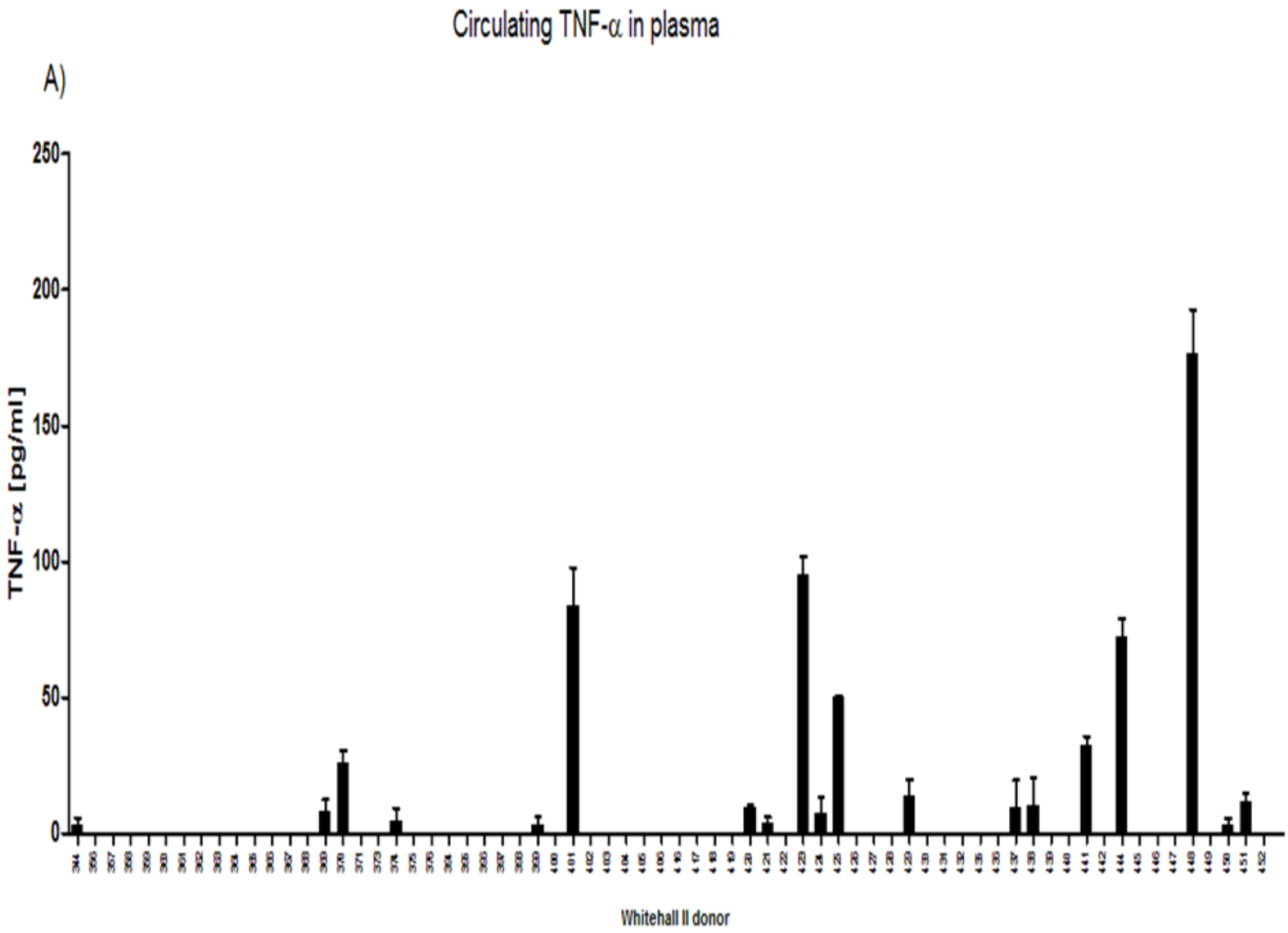
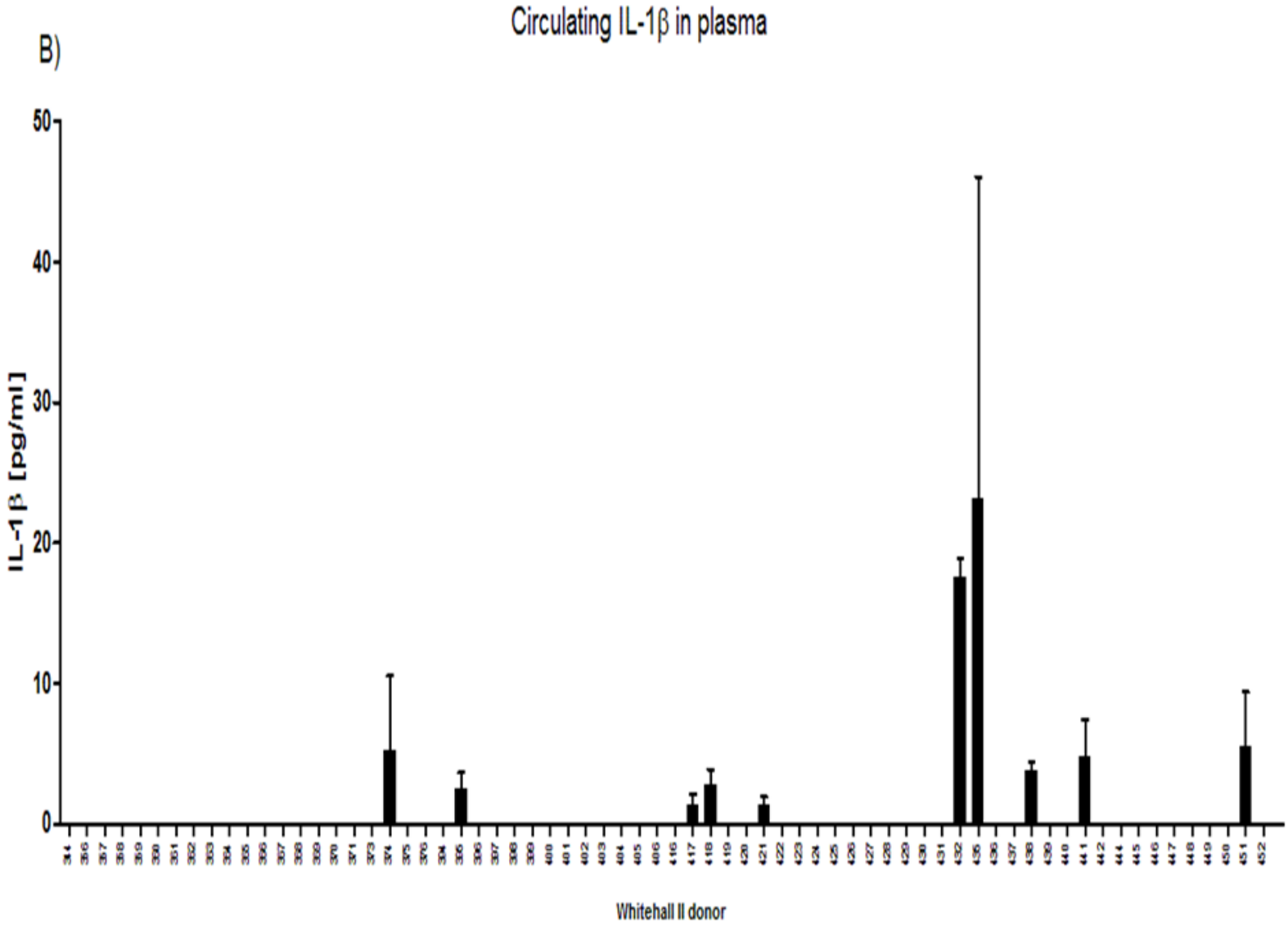

D)
Circulating TGF-β in plasma
TGF-β [pg/ml]
2500
2000
1500
1000
500
0
Whitehall sample

Figure 3.2 Variability of circulating TNF-α, IL-1β, IL-10 and TGF-β concentrations in plasma from the Whitehall II donors. Circulating TNF-α (A), IL-1β (B), IL-10 (C) and TGF-β (D) were measured using ELISA from plasma. Each bar represents the mean of triplicate measurements of one of 68 donors and the error bar represents the standard deviation. The range of circulating IL-1β varies from undetected to 35 pg/ml while the range of circulating TNF-α varies from undetected to 1000 pg/ml. The range of circulating IL-10 varies from 1 to 34 pg/ml while the range of circulating TGF-β varies from undetectable to 1723 pg/ml. Data for IL-1β, TNF-α and IL-10 were generated by Dr Frank Kaiser, a postdoctoral research associate working in the lab.


Circulating TNF-α in plasma
A)
TNF-α [pg/ml]
250
200
150
100
50
0
Whitehall II donor


B)
Circulating IL-1β in plasma
IL-1β [pg/ml]
50
40
30
20
10
0
Whitehall II donor

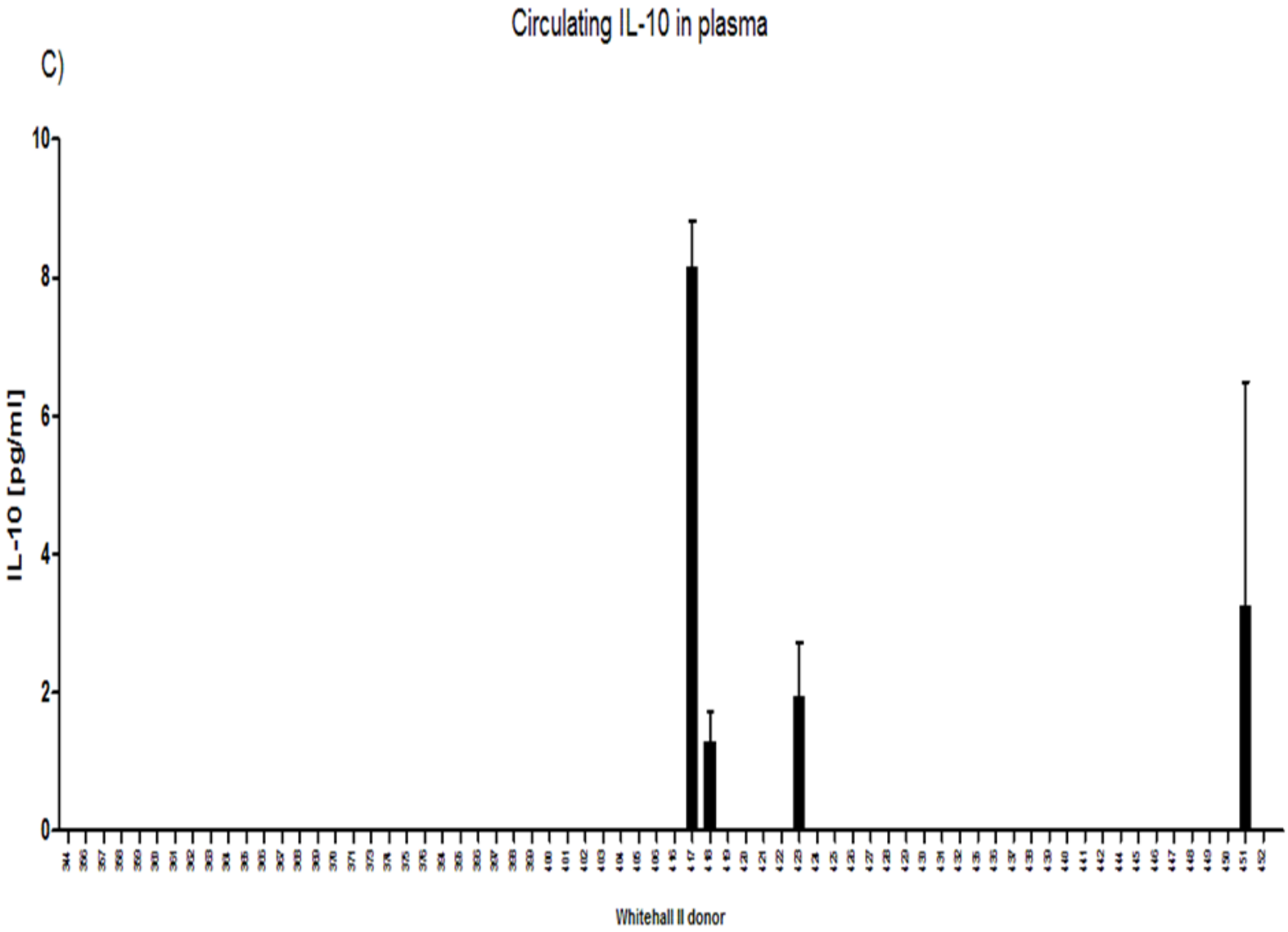

Circulating IL-10 in plasma
C)
IL-10 [pg/ml]
10
8
6
4
2
0
Whitehall II donor

Figure 3.3 Circulating cytokine levels in plasma of the Whitehall II donors was reduced after 2 years of storage at -20^{0}C. Circulating TNF-α (A), IL-1β (B) and IL-10 (C) concentrations were measured using ELISA. Each bar represents the mean of triplicate measurements of one of 68 donors and the error bar represents the standard deviation. The range of circulating IL-10 varies from undetectable to 8 pg/ml; TNF-α varies from undetectable to 176 pg/ml while the range of circulating IL-1β varies from undetectable to 23 pg/ml.

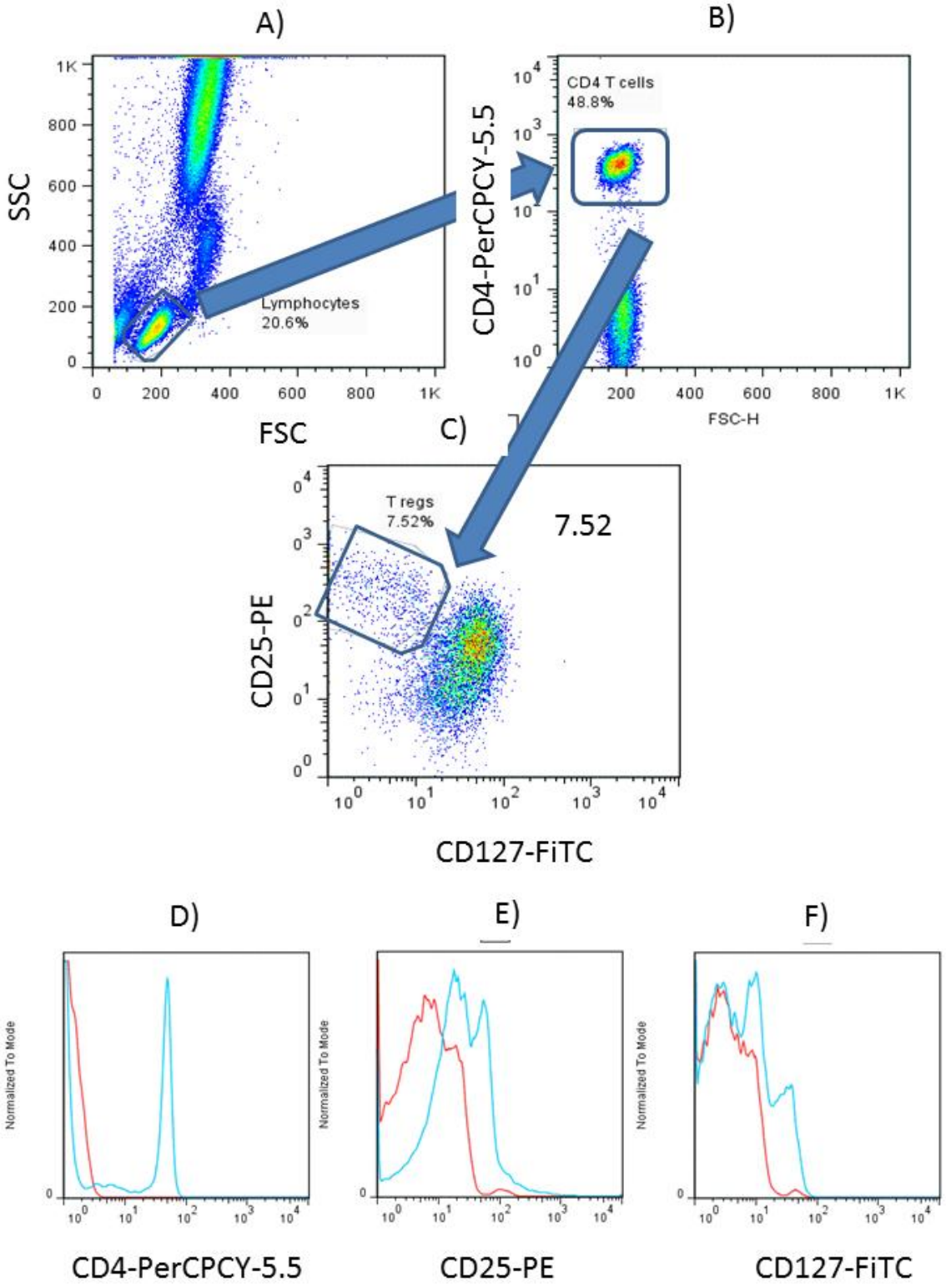

A)
B)
SSC
FSC
Lymphocytes
20.6%
CD4 T cells
48.8%
CD4-PerCPCY-5.5
FSC-H
C)
Tregs
7.52%
7.52
CD25-PE
CD127-FiTC
D)
E)
F)
Normalized To Mode
CD4-PerCPCY-5.5
CD25-PE
CD127-FiTC

Figure 3.4 Gating strategy employed to measure regulatory T cell frequency from whole blood. 50 µl of peripheral whole blood was stained with anti-CD4, anti-CD25 and anti-CD127 antibodies. After incubation with antibodies, cell suspensions were washed and fixed before data was acquired using FACS Calibur. Samples were acquired & recorded based on 20,000 events in the lymphocyte gate (A). After that, the lymphocyte gated sample was further re-gated according to CD4 expression (B). Finally, CD4 positive cells were gated against CD25 & CD127 expression (C). Regulatory T cell population can be identified as $CD4+CD25^{High}CD127^{Low}$ cells. Panel (D-F) show histograms comparing between antibody staining (blue line) to isotype control (red line) for CD4 (D), CD25 (E) and CD127 (F).

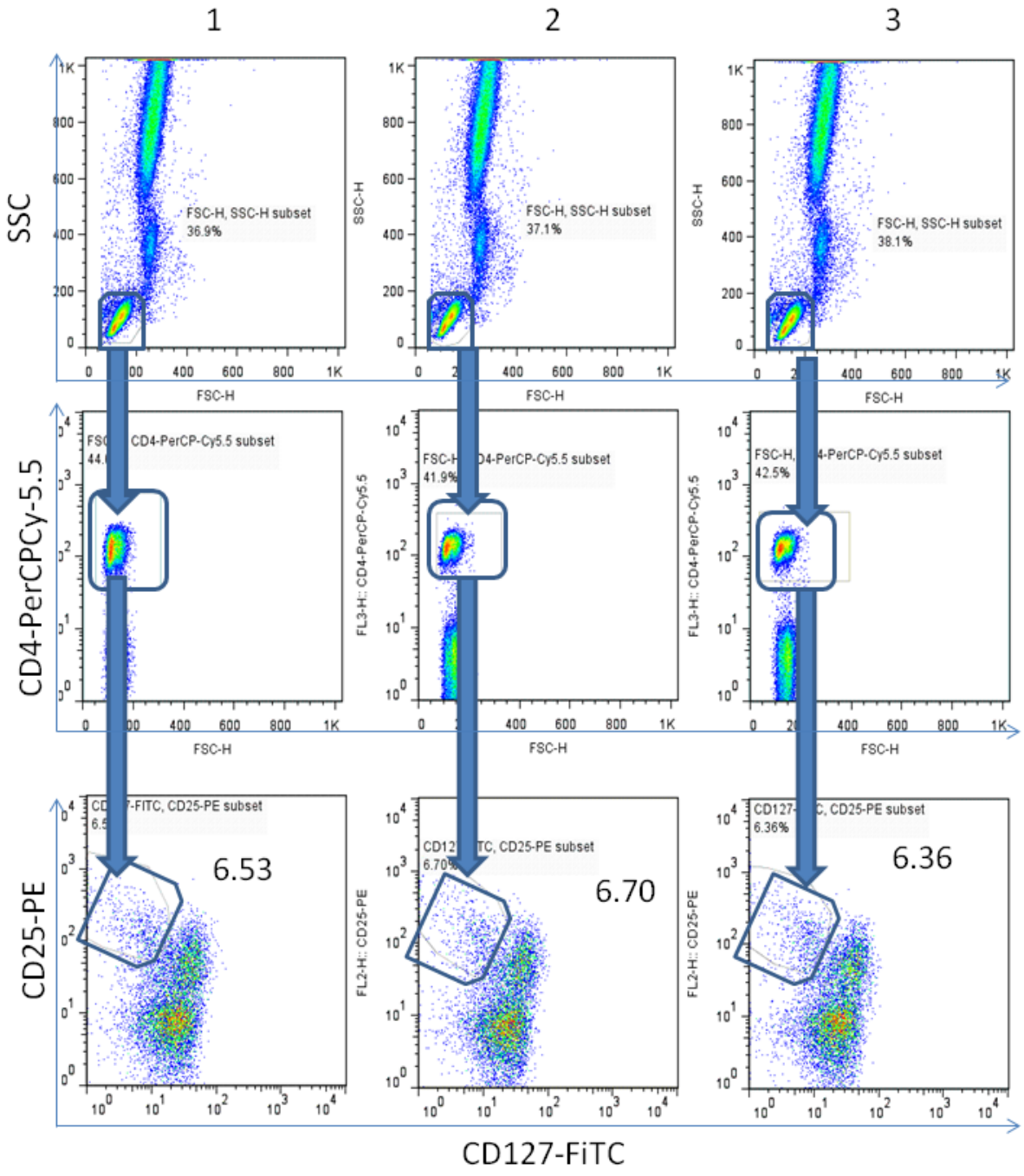
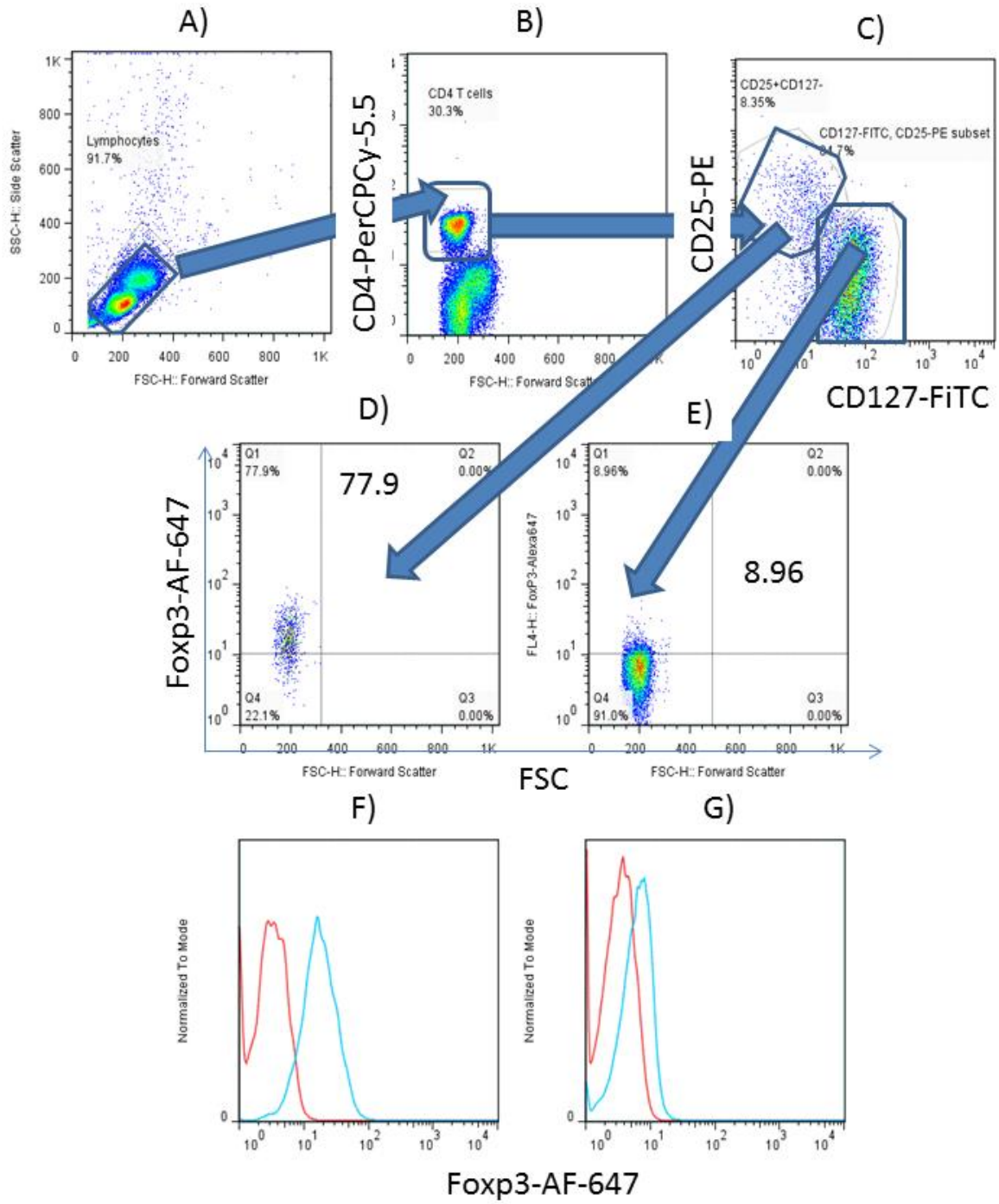


Figure 3.5 Triplicate measurements from one donor show similar frequencies of regulatory T cells. Regulatory T cell frequency was measured from whole peripheral blood and acquired using flow cytometry and analysed at the same time. The gating strategy used was as shown in Figure. 3.4. Triplicate measurements of regulatory T cell frequency from one donor are as follows; 6.53% (1), 6.70% (2) and 6.36% (3). These data are a representative of three donors.



(n=3)	Regulatory T cell $CD4^{+}CD25^{High}CD127^{Low}$	Responder T cell $CD4^{+}CD25^{Low}CD127^{High}$
Foxp3+ cells	75±9	12±8

Figure 3.6. The majority of CD4+CD25^{High}CD127^{Low} cells are FOXP3 positive. PBMCs were obtained by density gradient centrifugation using Lymphoprep. Surface staining was performed using anti-CD4, anti-CD25 and anti-CD127 antibodies. After that, intracellular staining of FOXP3 was performed. Sample was gated based on (A) Lymphocyte gate followed by (B) CD4 positive cells (C) CD25 versus CD127 expression. Panel (D) shows Foxp3 expression of CD4+CD25^{High}CD127^{Low} while panel (E) illustrates Foxp3 expression of CD4+CD25^{Low}CD127^{High}. Panel (F-G) show histogram comparing between Foxp3 antibody staining (blue line) versus isotype control (red line) of CD4+CD25^{High}CD127^{Low} (F) and CD4+CD25^{Low}CD127^{High} (G). The table records the mean percentage of Foxp3+ cells and standard deviation in multiple donors (n=3).

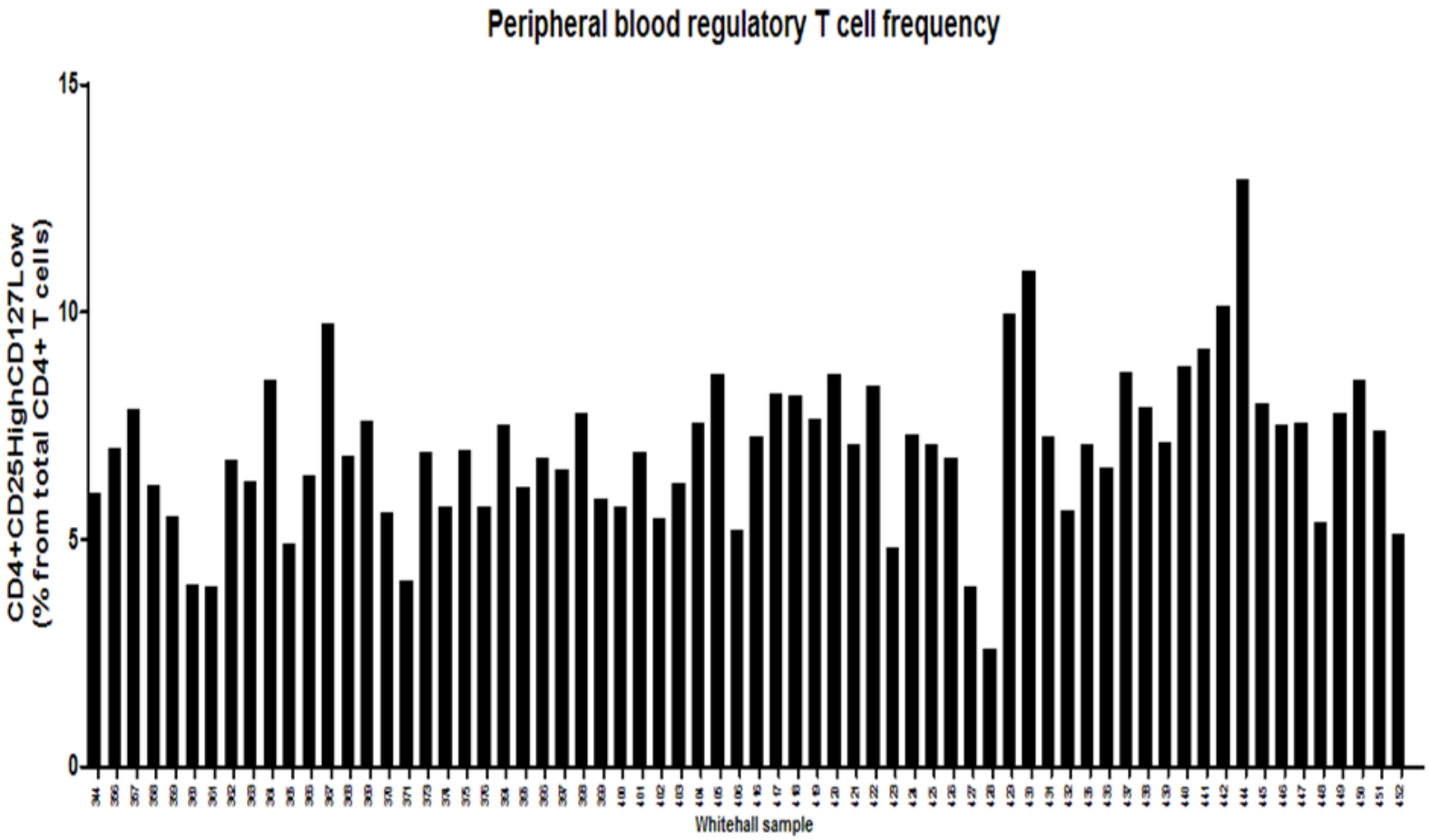


Figure 3.7 Regulatory T cell frequency measured in the Whitehall II donors. Regulatory T cell frequency was measured in whole peripheral blood using flow cytometry. The gating strategy employed to define regulatory T cells is shown in Figure 3.4. Each bar represents the regulatory T cell frequency measured in one of 68 donors of the Whitehall II study. The range of regulatory T cell frequency varies from 2.59% to 12.9% of total CD4+T cells with a mean of 6.9% regulatory T cell frequency.

	Reg.T cells	Res. T cells	Ratio	CD4+T cells	BiP	IL-1β	IL-10	TNF-α	TGF-β
Reg. T cells	1	-0.742	-0.017	-0.998	0.006	-0.122	-0.098	0.06	-0.044
Res. T cells	-0.742	1	0.149	0.735	0.097	-0.011	0.124	-0.038	-0.005
Ratio	-0.017	0.149	1	-0.055	-0.048	-0.164	-0.031	-0.085	0.084
CD4+ T cells	-0.998	0.735	-0.055	1	-0.008	0.112	0.093	-0.067	0.04
BiP	0.006	0.097	-0.048	-0.008	1	0.271	0.356	0.767	0.005
IL-1β	-0.122	-0.011	-0.164	0.112	0.271	1	0.352	0.332	0.109
IL-10	-0.102	0.124	-0.031	0.093	0.356	0.352	1	0.372	0.268
TNF-α	0.06	-0.038	-0.085	-0.067	0.767	0.332	0.372	1	-0.008
TGF-β	-0.044	-0.005	0.084	0.04	0.005	0.109	0.268	-0.008	1

Table 3.1. Soluble BiP correlates with most of the cytokines; but not with any cell frequency measurement. The value shown in each box is the coefficient correlation value (R) and indicates the nature of correlation either linear (+) or inverse (-). Matching data sets with Gaussian distribution was correlated using Pearson correlation test whilst data with a non-Gaussian distribution was correlated using Spearman correlation test. Correlations with $p<0.05$ was taken as statistically significant and marked in red colour while cells in orange colour indicate non significant correlations.

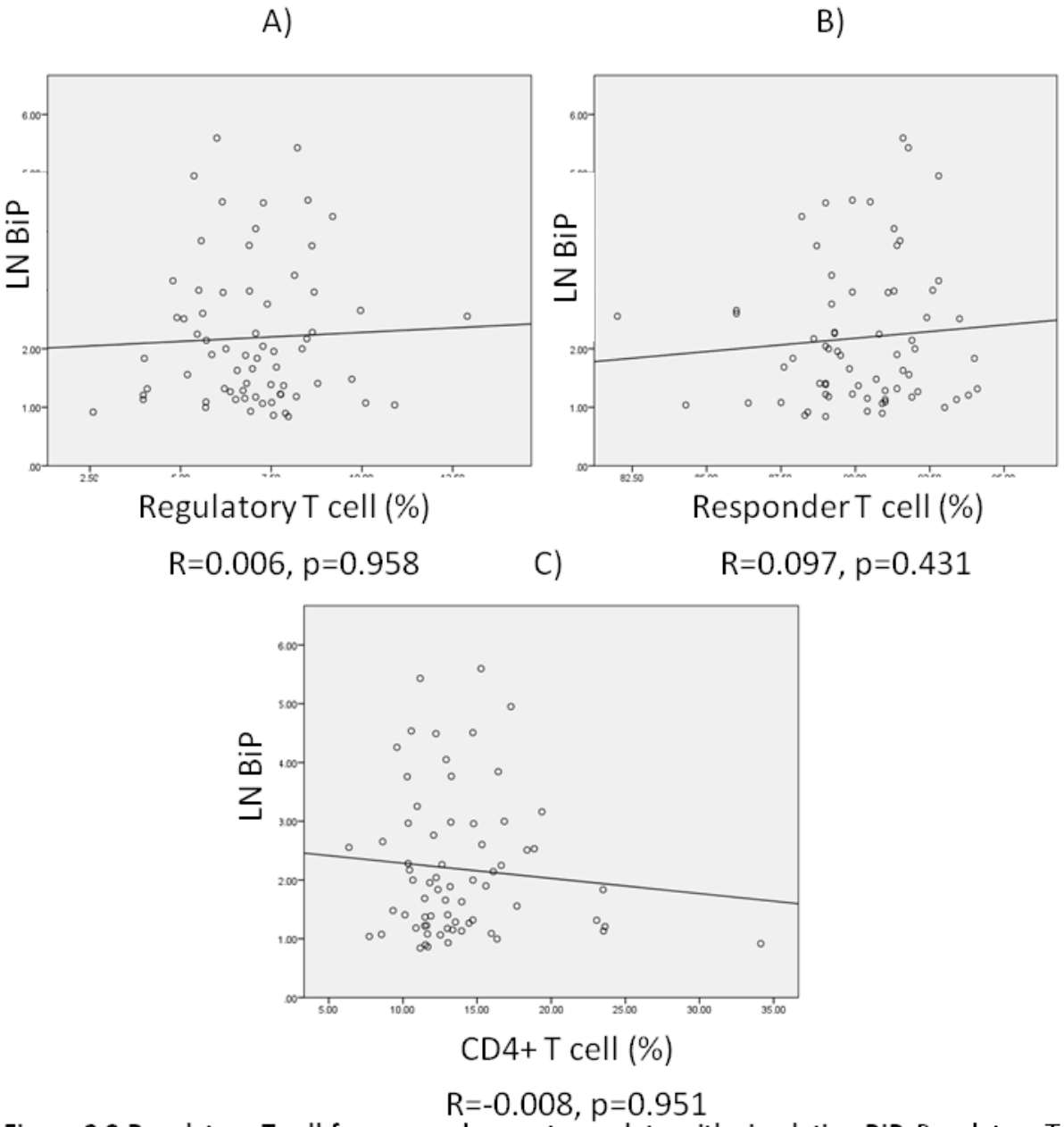


Figure 3.8 Regulatory T cell frequency does not correlate with circulating BiP. Regulatory T cell frequency was measured from whole peripheral blood staining using flow cytometry while circulating BiP and cytokine concentrations were measured using ELISA. Each dot represents one of 68 donors in the Whitehall II cohort. Panel (A-C) show the scatter plot of circulating BiP and regulatory T cell frequency (A), responder T cell frequency (B) and CD4+ T cell frequency (C). R value represents correlation coefficient where a positive R value indicates linear correlation whilst a negative R value demonstrates an inverse correlation. P<0.05 indicates the correlation to be statistically significant. LN indicates natural log.

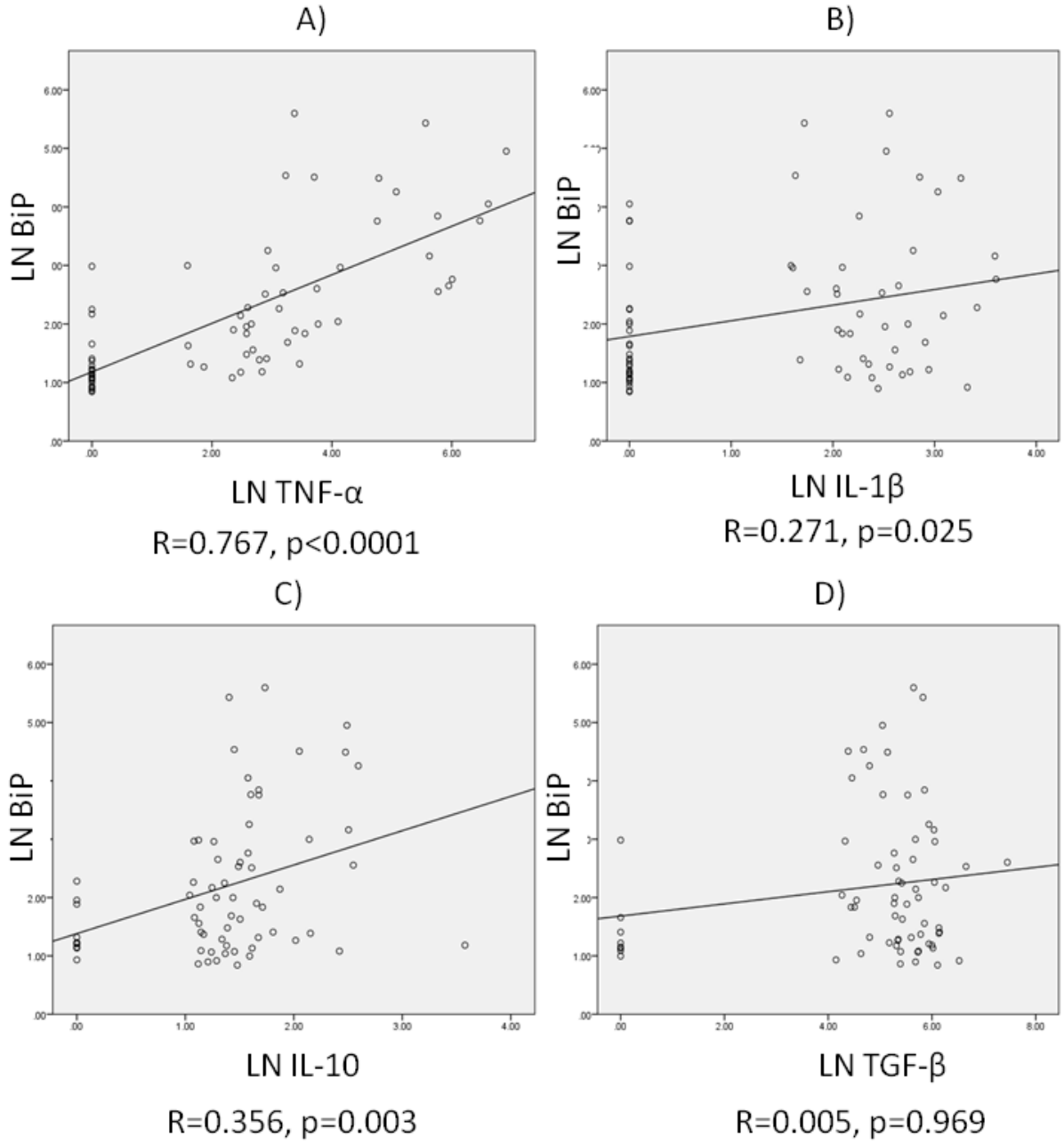


Figure 3.9 Circulating BiP levels correlate with most cytokines. Circulating BiP and cytokine concentrations were measured using ELISA. Each dot represents one of 68 donors in the Whitehall II cohort. Panel (A-D) show the scatter plot of circulating BiP and TNF-α (A), IL-1β (B), IL-10 (C) and TGF-β (D). R value represents correlation coefficient where a positive R value indicates linear correlation whilst a negative R value demonstrates an inverse correlation. P<0.05 indicates the correlation to be statistically significant.

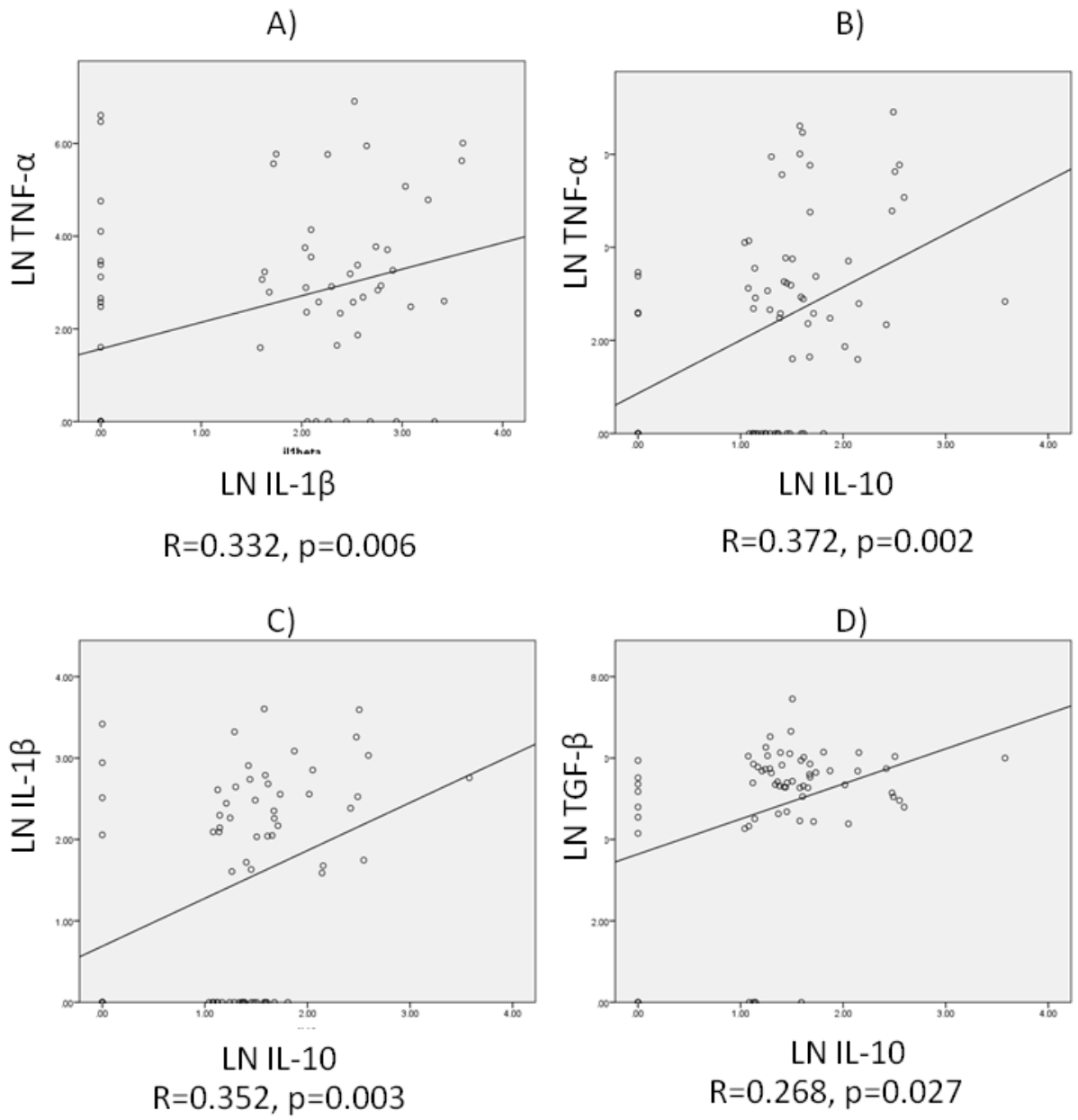


Figure 3.10 Circulating cytokine levels correlate with each other. Circulating cytokine concentrations were measured using ELISA. Each dot represents one of 68 donors in the Whitehall II cohort. Panel (A-D) show the scatter plot of circulating TNF-α and IL-1β (A), TNF-α and IL-10 (B), IL-1β and IL-10 (C) and TGF-β and IL-10 (D). R value represents correlation coefficient where a positive R value indicates linear correlation whilst a negative R value demonstrates an inverse correlation. P<0.05 indicates the correlation to be statistically significant.

Category	Parameter	Range
Anthropometric measures	Weight (kg)	45-104.3
	BMI (kg/m2)	17.19-40.20
Blood pressure	MSBP (mmHg)	90-181
	MDBP (mmHg)	54-107
Lipid profile	Total cholesterol (mM/L)	3.7-7.8
	Triglycerides (mM/L)	0.5-3.4
	HDL (mM/L)	1.1-3.2
	LDL (mM/L)	1.4-5.3
	Pericardial fat (Hounsfield unit)	45.34-276.23
Others	Glycated Haemoglobin (%)	4.8-8.2
Coronary artery calcification	Vol_tut (Agatston Unit)	0-1341.4
	Agot_tut(Agatston Unit)	0-1705.18

Table 3.2 The list of clinical/biochemical parameters measured in the Whitehall II study cohort. Clinical/biochemical parameters were measured and obtained from Prof. Andrew Steptoe, Department of Epidemiology & Public Health, UCL.

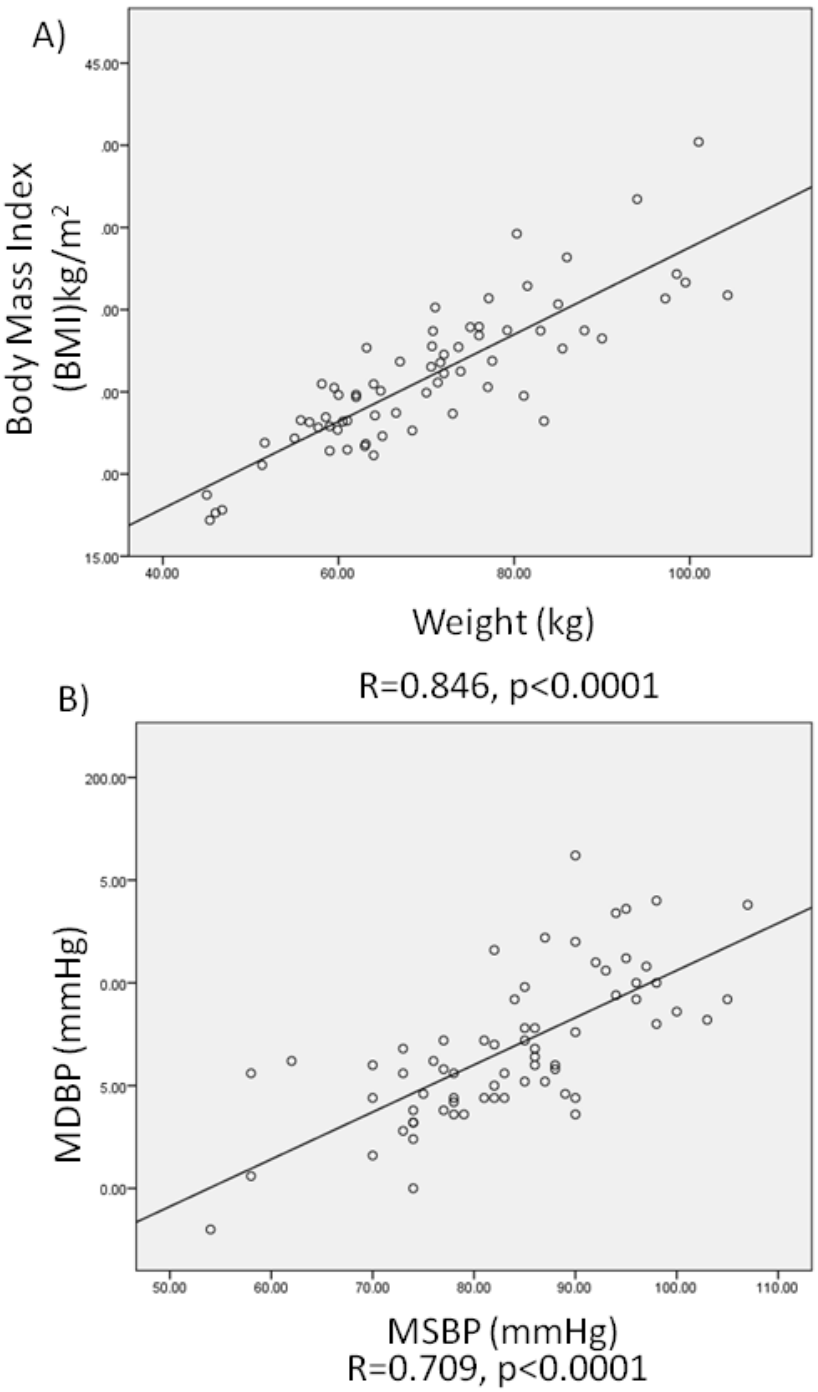


Figure 3.11 Correlation analyses validated by related parameters. All measurements were provided by our collaborator, Prof. Andrew Steptoe from UCL. Each dot represents one of 68 donors in the Whitehall II cohort. Panel (A) shows the scatter plot of body weight versus BMI while (B) demonstrates MSBP versus MDBP. R value represents the correlation coefficient. $p<0.05$ indicates correlation is statistically significant.

Significant correlation	R value	P value
Regulatory T cell frequency & Body weight	-0.246	0.043
IL-10 & Body weight	0.256	0.035
IL-10 &Pericardial fat	0.264	0.03
BiP & Triglycerides	-0.265	0.029

Table 3.3. Significant correlations were observed between various immunological parameters and clinical parameters. Matching data sets with Gaussian distribution were correlated using Pearson correlation test whilst data with non-Gaussian distributions were correlated using Spearman correlation test. R value represents correlation coefficient with positive R value indicates linear correlation while a negative R value demonstrates inverse correlation. $p<0.05$ indicates correlation is statistically significant.

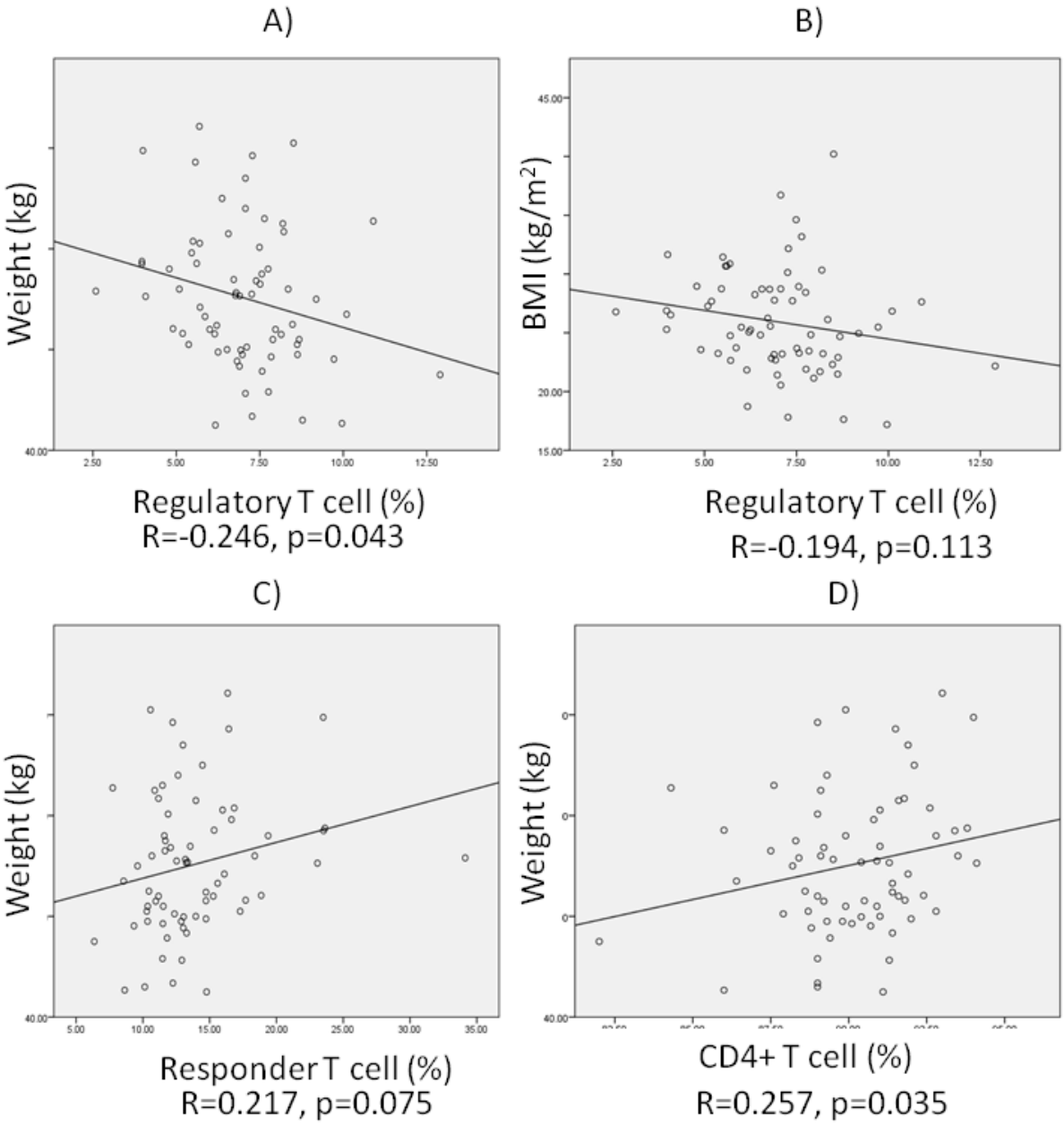


Figure 3.12 Regulatory T cell frequency correlates with body weight. Regulatory T cell frequency was measured by flow cytometry while circulating BiP concentrations were measured using ELISA in plasma of the Whitehall II cohort. Body weight was measured by our collaborator, Prof. Andrew Steptoe from UCL. Each dot represents one of 68 donors in the Whitehall II cohort. Panel (A-C) shows scatter plot of weight versus regulatory T cell frequency (A), responder T cell frequency (B) and CD4+ T cell frequency (C). R value represents correlation coefficient with a positive R value indicating linear correlation while a negative R value demonstrates an inverse correlation. $p<0.05$ indicates correlation is statistically significant.

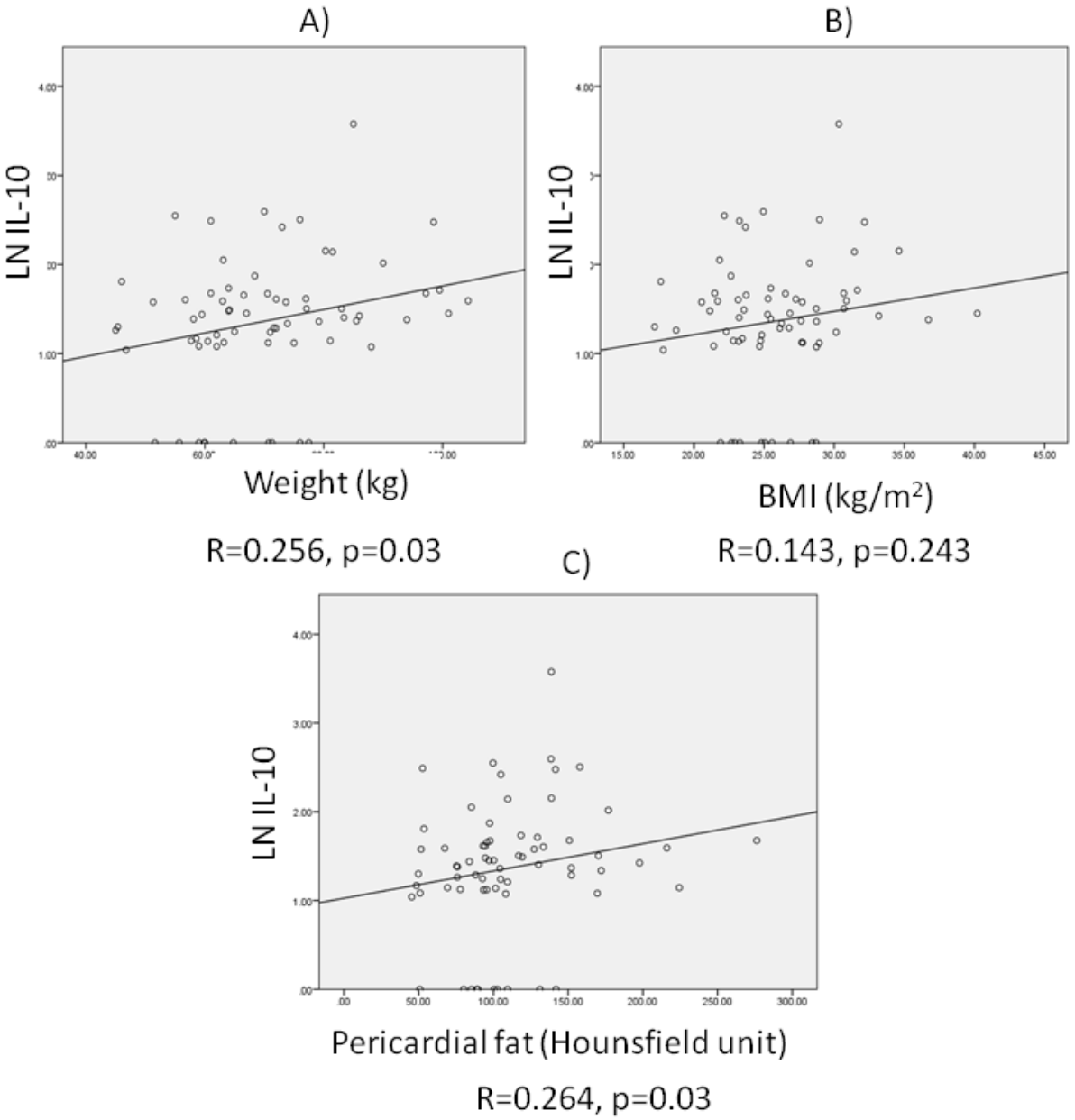


Figure 3.13 Circulating IL-10 levels correlate with body weight and pericardial fat. Circulating IL-10 concentrations were measured using ELISA in plasma of the Whitehall II cohort. Body weight and pericardial fat were measured by our collaborator, Prof. Andrew Steptoe from UCL. Each dot represents one of 68 donors in the Whitehall II cohort. Panel (A) shows scatter plot of circulating IL-10 concentrations versus body weight while (B) demonstrates circulating IL-10 concentrations versus triglycerides. R value represents correlation coefficient where a positive R value indicates linear correlation whilst a negative R value demonstrates an inverse correlation. $P<0.05$ indicates the correlation to be statistically significant.

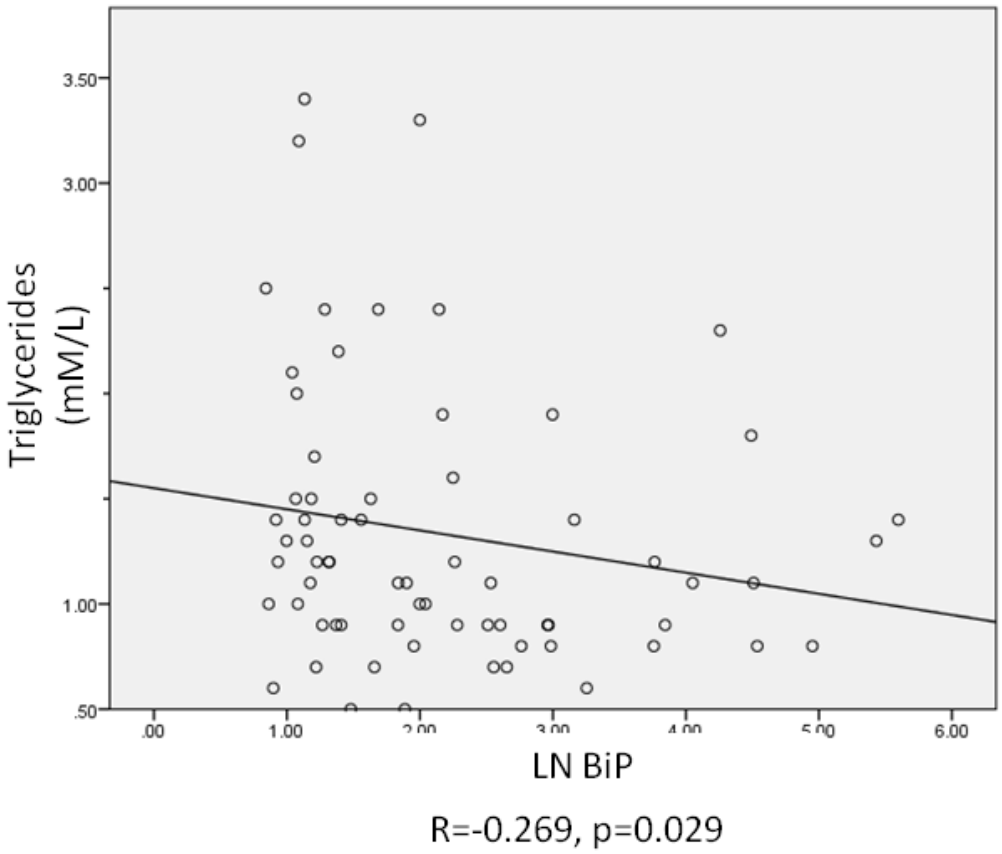


Figure 3.14 Circulating BiP levels correlate with triglycerides. Circulating BiP concentrations were measured using ELISA in plasma of the Whitehall II cohort. Triglyceride concentrations were measured by our collaborator, Prof. Andrew Steptoe from UCL. Each dot represents one of 68 donors in the Whitehall II cohort. The figure (A) the shows scatter plot of circulating BiP levels versus triglyceride concentrations. R value represents correlation coefficient where a positive R value indicates linear correlation whilst a negative R value demonstrates an inverse correlation. $P<0.05$ indicates the correlation to be statistically significant.

3.4 Discussion

3.4.1 Regulatory T cell frequency does not correlates with circulating BiP nor circulating cytokine concentrations

Data obtained from these correlation analyses performed on various parameters should be carefully interpreted. Based on Table 3.1 and Table 3.3, most of the correlations are weak correlations as denoted by low R value of less than 0.5. Therefore, these correlations must be supported or validated by other epidemiological or mechanistic studies. Furthermore, the size of the sample used for the correlation analysis is small (n=68) and the conclusion inferred from the correlation analysis cannot be applied to the general population as the mean age of this cohort is 62.2 years. Furthermore, these data were obtained from a cohort of healthy individuals; therefore the conclusions inferred from the correlation analysis cannot be applied to people with pathological conditions such as cardiovascular disease and diabetes mellitus.

The main focus of this chapter is to investigate the relationship between regulatory T cell frequencies and circulating BiP. Previous work by Shields et al (2011) showed that concentration of circulating soluble BiP in the range of ng/ml either in sera of non-rheumatoid arthritis or rheumatoid arthritis subjects. This discrepancy may be due to several factors. Circulating BiP detected in this assay involves full length BiP that may be released into the circulation and soluble BiP whereas previous work by Shields and co-workers (2011) only detected soluble BiP (lacks KDEL sequence). In addition, it may be possible that the assay can detect BiP in free form or antibody-bound form. Another difference is that BiP measurement was performed from plasma samples while the latter was performed in sera. Donors with very high level of circulating BiP concentration such as donor 344 (268 µg/ml), donor 369 (227 µg/ml) and donor 448 (140 µg/ml) may have been experiencing subclinical infection or other pathologies when the blood was taken. Viral infection has been reported to induce an increase in BiP expression. For instance, human cytomegalovirus infection which causes few symptoms in most people can induce an increase in BiP transcription and translation as reported by Buchkovich and colleagues (2010).

Correlation analyses revealed that association between regulatory T cell and BiP is statistically insignificant (Table 3.1 and Figure 3.8(A)). These data are in concordance with correlation of circulating regulatory T cells with HSPs as shown by Dempsey and co-workers (2010). In their study, they demonstrated that no correlation was observed between regulatory T cell frequency and HSP27, 72 and 90 in both chronic lymphocytic leukemia patients and normal healthy controls. Non-significant correlations between BiP and regulatory T cells can be partly explained by the fact that all nucleated cells express BiP (Zimmerman & Dudek, 2009); therefore BiP present in the circulation is not limited to BiP secreted by regulatory T cells only. No association between regulatory T cell frequency and all cytokines can be explained by the same fact that cytokine secretion is not limited to regulatory T cells only (Table 3.1). IL-10 can be secreted by other immune cells such as DC, macrophages, and T cells (Saraiva & O'Garra, 2010) while other cells such as platelet (Grainger et al, 2000); lymphocytes, macrophage and dendritic cells can secrete TGF-β (Letterio & Roberts, 1998) hence no association between regulatory T cell frequency and IL-10 or TGF-β.

3.4.2 Regulatory T cell frequency negatively correlates with body weight; not with other clinical/biochemical parameters

As shown in Table 3.3 and Figure 3.12(A), circulating regulatory T cell frequency negatively correlates with body weight and this association is in concordance with Wagner et al (2013). Their study reported a negative correlation between regulatory T cell frequency and body weight, and the data from the Whitehall II cohort reported the same correlation. However, they reported that circulating regulatory T cell frequency negatively correlates with BMI; not from our analysis (Figure 3.12(B)). The discrepancy between the data and their result because of different population used as regulatory T cell frequency. They defined circulatory regulatory T cell frequency as $CD4^{+}CD25^{High}CD127^{Low}Foxp3^{+}$ population while $CD4^{+}CD25^{High}CD127^{Low}$ population is defined as regulatory T cells in the study. Therefore, the data demonstrate non-significant correlation between regulatory T cell frequency and BMI (R=-0.194, p=0.113).

To further support these data, correlation analyses were performed on CD4+ T cells and body weight as illustrated in Figure 3.12(D). The data indicate positive correlation between CD4+ T cells and body weight (R=0.257, p=0.035) and these data are supported by

van der Weerd et al (2012) where they showed increased CD4+ T cells in morbidly obese subjects. Circulating regulatory T cell negative correlation with body weight could be partly explained by the effect of leptin on regulatory T cell function. Leptin is a hormone involved in the control of appetite and obesity and manufactured primarily in the adipocytes of white adipose tissue. High levels of leptin have been associated with obese people as reviewed by Ahima (2008). An interesting observation was made by Matarese and co-workers (2005) where they observed a negative correlation between serum leptin and CD4+CD25+ regulatory T cells in a group of multiple sclerosis patients. De Rosa et al (2007) further demonstrated that leptin can negatively regulate regulatory T cells by inducing anergy and unresponsiveness when leptin binds to leptin receptor on regulatory T cells themselves. In contrast to regulatory T cells, leptin acts in a positive feedback on CD4+CD25- effector T cells by further encouraging effector T cell proliferation. However, to further confirm this observation, an accurate measurement of regulatory T cell could be performed by measuring regulatory T cell count instead of frequency on more donors (n>90).

Despite various literature suggesting the important role played by regulatory T in reducing atherosclerosis, correlation analyses revealed regulatory T cell frequency association with body weight only; not with any clinical/biochemical parameters. Ammirati and colleagues (2010) demonstrated that circulating regulatory T cells do not reflect the severity of atherosclerosis in patients with the history of CVD events. In addition, most of the studies about regulatory T cells in animal model of atherosclerosis focus on regulatory T cells present in atherosclerotic plaques and its function in reducing atherosclerosis; while this study measured circulating regulatory T cell frequencies in the circulation. A study performed by Kyriakakis and colleagues (2010) demonstrated the presence of invariant natural killer T (iNKT) cells in human atherosclerotic plaque and they reported that iNKT cells were diminished in the circulation of symptomatic atherosclerosis patients. Therefore, iNKT frequency is a better alternative than regulatory T cell frequency for association studies with established risk factors of CVD.

3.4.3 Circulating IL-10 levels correlate with body weight and pericardial fat suggesting that IL-10 may be used as a marker of systemic inflammation

IL-10 is one of the cytokines measured in plasmas from the cohort. Correlation analysis revealed IL-10 association with body weight (R=0.256, p=0.035) and pericardial fat (0.264, p=0.03) as demonstrated in Table 3.3 and Figure 3.13 (A&C). Circulating IL-10 cytokine correlation with body weight may be explained by 2 factors; reaction to low, chronic grade of inflammation and the fact that obese individuals are more prone to infection. Obesity is associated with low, chronic inflammation. Adipocytes play a contrasting role in lean versus obese individuals. In consensus, lean adipose tissue contains regulatory immune cells such as reg. T cells, Th2 cells, M2 macrophage and eosinopils that suppress inflammatory immune cells (Han & Levings, 2013) in lean individuals while inflammatory immune cells including Th1 cells, M1 macrophages, CD8+ T cells, B cells, dendritic cells, mast cells and neutrophils dominate in obese adipocytes. In addition, Fabbrini and colleagues (2013) reported an increased frequency of Th17 and Th22 phenotype in adipocytes that can secrete IL-17 and IL-22 in obese individuals compared to lean subjects. These 2 cytokines are associated with pro-inflammatory phenotype and contributes to insulin resistance in obese individuals. Furthermore, inflammatory condition in obese visceral adipose tissue is worsened by adipocyte secretion. Yehuda-Shnaidman and Schwartz (2012) compared different soluble factors secreted by adipose tissue from lean and obese visceral adipose tissue and clearly adipocytes from obese individuals secreted more pro-inflammatory factors such as TNF-α, leptin, IL-1β, IL-6 and IL-8 and reduced anti-inflammatory factors, namely IL-10 and adiponectin. In various pathological conditions such as rheumatoid arthritis, the presence of pro and anti-inflammatory factors have been reported on the site of active inflammation suggesting that anti-inflammatory factors are secreted to counter act the effect of pro-inflammatory factors even though inflammation persists (Bogdan et al, 1992). Therefore, it is possible that the presence of IL-10 in the circulation is counter-acts the effect of pro-inflammatory factors secreted by adipocytes into the circulation.

Another reason to support the IL-10 association with body weight is that obesity is associated with infection. Supporting evidence from literature shows that obesity is an independent risk factor in infection. Saliba and co-workers (2013) showed that obesity is

independently associated with urinary tract infection especially in males. *Clostridium difficile* infection, a type of bacterial infection affecting digestive system, is also prominent in obese hospitalised patients in a study conducted by Bishara et al (2013). In addition, obesity has also been shown to influence viral infection. A cohort study in the Swedish population showed that obesity in children and adults is associated with adenovirus infection as demonstrated by Almgren et al (2012). Following the H1N1 outbreak worldwide in 2009, van Kerkhove and colleagues (2011) published a report to identify risk factors associated with H1N1 infection. One of the risk factors associated with H1N1 infection is obesity especially for people with BMI of 30 or more. Based on this evidence, Kanneganti & Dixit (2012) proposed defective immunological surveillance as one of the consequences of obesity on the immune system. Therefore, obese individuals are more prone to infection compared to lean individuals. We proposed that the obesity association with infection is due to higher circulating IL-10 compared to people with normal BMI. By culturing PBMC with IL-10 cytokine, Levitz et al (1996) indicated that TNF-α & IL-1β secretions are significantly reduced when PBMC were simulated with LPS, *Candida albicans* or *Cryptococcus neoformans*. When PBMCs are infected with LPS, *C.albicans* or *C.neoformans*, TNF-α and IL-1β secretions upon challenge with LPS, *C.albicans* or *C.neoformans* are important to promote inflammation to help clearing up the infection. Furthermore, Malavige et al (2013) demonstrated that IL-10 contributes in inhibiting IFN-γ response in dengue peptide specific T cells in dengue viral infection. Therefore, this evidence supports IL-10 association with body weight.

In addition, circulating IL-10 correlates with another risk factor of CVD which is pericardial fat ($R=0.269$, $p=0.027$) (Table 3.3 and Figure 3.13 (C)). Correlation analysis from the cohort reveals that pericardial fat is strongly associated with other CVD risk factors such as weight ($R=0.647$, $p<0.0001$), triglycerides ($R=0.448$, $p<0.0001$) and BMI ($R=0.531$, $p<0.0001$) (not shown). Pericardial fat has been identified as a CVD risk factor either independently (Mahabadi et al, 2009, Yun et al, 2012, and Miao et al, 2011) or dependently on other CVD risk factors (Greif et al, 2013 and Lee et al, 2014). Our association is indirectly supported by an epidemiological study conducted by Welsh and colleagues (2011). They showed that circulating IL-10 negatively correlates with HDL and positively correlates with IL-6 and CRP, both factors associated with CVD risk factors. In addition, they demonstrated

higher IL-10 in the circulation of people with CVD events. These associations were not expected as the role of IL-10 in arterial plaques has been shown to be anti-inflammatory, not pro-inflammatory. Therefore, a negative association is expected between circulating IL-10 and CVD risk factors. Furthermore, Lakoski and co-workers (2008) demonstrated that elevated IL-10 concentration was associated with increased risk for future cardiovascular events in post-menopausal women with established coronary atherosclerosis.

One of the potential mechanisms of action explaining the positive association between IL-10 and pericardial fat is that IL-10 secretion is used to counter deleterious effect of pro-inflammatory mediators released by pericardial fat. Mazurek and colleagues (2003) demonstrated that epicardial fat, one component of pericardial fat as argued by Carr & Ding (2009), is a source of inflammatory mediators. They showed that epicardial fat expressed higher mRNA and protein levels of IL-1β, IL-6, MCP-1 and TNF-α compared to subcutaneous fat. To counter the effect of these pro-inflammatory mediators, IL-10 is an essential cytokine since Lumeng et al (2007) showed that IL-10 protects adipocytes from the deleterious effect of MCP-1 and TNF-α. IL-10 treatment on adipocytes reduced MCP-1 secretion from adipocytes by antagonising NF-κB-mediated gene transcription. In addition, IL-10 can protect adipocytes from TNF-α-induced downregulation of insulin receptors and glucose transported 4 expressions; both of which molecules are important for normal physiological function. Hence, circulating IL-10 has been suggested not only as a marker to be used for predicting CVD but also as a surrogate marker for ongoing systemic inflammation and related pathophysiological processes as suggested by Welsh et al (2011).

3.4.4 Circulating BiP concentrations correlate with most cytokines and triglycerides

In contrast to regulatory T cell frequency, circulating BiP concentration correlates with 3 of 4 circulating cytokines measured namely TNF-α ($R=0.767$, $p<0.0001$), IL-1β ($R=0.271$, $p=0.025$), and IL-10 ($R=0.356$, $p=0.003$); but not with TGF-β($R=0.005$,$p=0.969$) as shown in Table 3.1 and Figure 3.9. TGF-β measured in plasma can be contributed by the secretion from non-immune cells. In addition to TGF-β secretion by leukocytes, Grainger et al (2000) argued that plasma TGF-β contributed partly by *in vivo* platelet degranulation. Platelets are small, disk shaped clear cell fragments derived from megakaryocytes and most importantly have no nuclei. Non-significant associations between BiP and TGF-β can be partly explained by the fact that BiP can only be expressed and secreted by nucleated cells;

therefore BiP could not be expressed or secreted by platelets. However, evidence from literature supports BiP association with other cytokines measured.

IL-1β and TNF-α are both pro-inflammatory cytokines involved in early stages of inflammation. Various articles suggest that these cytokines can stimulate various cell types to upregulate BiP expression. Yoo et al (2012) showed that culturing human synoviocytes, a specialised cell type located inside joints in the synovium, with TNF-α and IL-1β can increase BiP expression. In addition, Verma and Datta (2010) demonstrated that incubating human pancreatic epithelial cells with IL-1β can induce ER stress markers including BiP. However, our correlation analysis reveals that TNF-α association with BiP is stronger compared to IL-1β association with BiP as denoted by higher R value. This can be explained by the fact that TNF-α secretion can also be induced by BiP upregulation but not with Il-1β. Using a human cancer cell line, Hu and colleagues (2006) showed that ER stress induction which upregulates BiP expression can lead to TNF-α secretion. ER stress induced IRE1α upregulation which subsequently lead to NF-κB activation and thus inducing TNF-α secretion from these cells. Furthermore, Lu and colleagues (2010) demonstrated that BiP can indirectly induce TNF-α production in human monocytes. TNF-α production is induced when anti-citrullinated protein antibodies bind to surface BiP on monocytes by enhancing NF-κB activity. This evidence leads to proposed cross talk between ER stress and innate immune system as suggested by Martinon and Glimcher (2011). However, the effect of ER stress, especially BiP upregulation is not limited to TNF-α secretion only.

The correlation analysis show that BiP correlates positively with IL-10. As a major anti-inflammatory cytokine, IL-10 secretion is essential to counteract the effect of pro-inflammatory cytokines including TNF-α and IL-1β. Bodman-Smith and co-workers (2003) showed that extracellular recombinant BiP protein can stimulate IL-10 production from CD8+ T cells from healthy individuals. Further work by the same group later demonstrated that PBMC cultured in the presence of recombinant BiP protein can secrete IL-10 (Corrigall et al, 2004). In the presence of BiP, PBMC cultured in the absence of any stimulus upregulated TNF-α after 7 hours but TNF-α production was reduced after 24 hours of culture. In contrast, IL-10 production was increased from 7 to 24 hours of culture and IL-10 production in culture was sustained until 96 hours of culture. IL-10 production in PBMC cultured with BiP was mainly produced by monocytes via the p38 MAPK pathway; not the

ERK pathway. This evidence supports the data that show association between circulating BiP and cytokines.

Circulating cytokines correlates with each other as shown in Table 3.1 and Figure 3.10. TNF-α and IL-1 correlates with each other (R=0.332, p=0.006) as both cytokines are pro-inflammatory cytokines. This correlation can be partly explained by the fact both cytokines can be induced by the same transcription factor. Both cytokines can be induced in cells after the activation of NFκB transcription factor as reviewed by Caamano and Hunter (2002). Interestingly, both cytokines correlates with IL-10 (R=0.372, p=0.002) (R=0.352, p=0.003), the major anti-inflammatory cytokine. TNF-α and IL-1β are some of the cytokines involved in the early stage of immune response since these two cytokines can be secreted by innate immune cells. However, IL-10 as major anti-inflammatory cytokine can be released in the later stage of immune response to counter act the effect of pro-inflammatory cytokines and restore homeostasis. Wang and co-workers (1995) demonstrated that IL-10 can block the induction of pro-inflammatory cytokines such as TNF-α and IL-1β in human monocytes by blocking the activation of NFκB transcription factor. This could partly explain the positive correlation between IL-10 and TNF-α & IL-1β. On the other hand, IL-10 positively correlates with TGF-β, a pleiotropic cytokine. IL-10 and TGF-β had been reported to act together in anti-tumour immunity (Jarnicki et al, 2006), and the induction of regulatory T cells (Chen et al, 2003). Most importantly, Huss and colleagues (2011) demonstrated that TGF-β signalling in murine Th1 cells can induce IL-10 secretion via SMAD4 transcription factor.

Significant correlation between different cytokines may be because of false positive results. Donors with high circulating cytokine levels may record higher cytokine concentrations than the actual concentrations because of the interference of heterophilic antibodies. Heterophilic antibodies are defined as antibodies that can be induced by external antigens. Todd and co-workers (2011) reported that heterophilic antibodies can interfere with cytokine and chemokine measurements including TNF-α and IL-1β. An example of heterophlic antibody is rheumatoid factor (RF). It is an autoantibody against the Fc portion of immunoglobulin (IgG) and normally can be used in the diagnosis of RA. However, Kyburz et al (1999) reported that RF can be detected in up to 30% of normal individuals. Therefore, it is possible that the cytokines measured by ELISA could be detecting

RF from these donors hence the correlation analyses performed showed significant correlation between various cytokines. The presence of heterophilic antibodies such as RF can be removed to ensure that the cytokine measurements are accurate. De Jager and colleagues (2005) compared between three different methods of removing heterophilic antibodies especially RF in human plasma samples. They showed that preabsorption of human plasma with protein-L was the best method to remove interfering antibodies. Preabsorption of plasma with protein-L can reduce RF IgM levels by 89% whereas total IgM, IgG and IgA levels were reduced by 60%. Therefore, future cytokine measurements should consider removing heterophilic antibodies to get accurate measurement of cytokines.

In addition, the presence of soluble cytokine receptors may interfere with the cytokine measurements even though Mosmann and Fong (1989) reported that cytokine ELISA are exquisitely specific because antibodies directed against two or more distinct epitopes. In addition, Callard and Gearing (1994) in their review mentioned about the role played by these receptors. These receptors may act as antagonists such as soluble TNF-α receptor or as carrier proteins *in vivo* and may serve as disease markers in *in vitro* tests. Furthermore, Carter and Swain (1997) demonstrated that cytokines detected by ELISA may or may not correlate directly with the levels of bioactive cytokine protein. For instance, an ELISA may utilize anti-cytokine antibodies that cannot discriminate between the precursor (inactive) and mature (bioactive) forms of a cytokine protein such as TGF-β. Moreover, an ELISA may detect partially-degraded cytokine proteins which have retained their immunoreactive propertiesbut may have lost their bioactivity. Cytokine sandwich ELISAs is useful indicators of the presence and levels of cytokines but they do not actually provide information concerning the biological potency of the detected proteins. Therefore, the correlation data between different cytokines may be validated by examining cytokine production in specific cell subset and specific stimulus as these two factors are important in cytokine production as reported by Ehlers and Smith (1991).

Correlation analyses were performed between circulating BiP and clinical/biochemical parameters. The only significant correlation was between circulating BiP and triglyceride concentrations from the cohort as shown in Table 3.3 and Figure 3.14. Indirect evidence shows a direct association between BiP and triglycerides, in contrast to the data. Werstuck et al (2001) demonstrated that BiP overexpression in human endothelial

cells can play a role in the activation of cholesterol/triglycerides biosynthesis. The discrepancy between the result and Werstuck et al (2001) could be partly explained by the system used in the experiment. The data obtained was looking at triglyceride levels in the circulation whilst Werstuck and co-workers (2001) investigated the effect of BiP on specialised in vitro culture system.

In conclusion, data obtained from correlation analysis can give a clue to the possible role of immunological parameters in predicting CVD. However, given that the size of the cohort is small (n=68) and this study is only limited to the elderly population, further mechanistic study could help to elucidate the exact role of each immunological parameter measured from this cohort. The data indicate associations between circulating BiP and cytokine concentrations suggesting cross talk between ER stress and immune system.

3.5 Conclusion

The main question of this chapter is to investigate the relationship between regulatory T cell frequency and circulating BiP concentration. Data obtained from the Whitehall II cohort revealed no association between regulatory T cell and BiP. Correlation analyses can explain relationship between two variables but cannot explain whether one variable can affect or modulate the function of the other variable. Therefore, the question remains whether BiP can affect or modulate regulatory T cell function and this question will be addressed in the next chapter.

Chapter 4

The effect of soluble BiP on regulatory T cell phenotype and function

4 The effect of soluble BiP on regulatory T cell phenotype and function

4.1 Introduction

In the previous chapter, data from the Whitehall II cohort showed that soluble BiP concentration does not correlate with regulatory T cell frequencies. Correlation analysis can only detect linear relationships between two variables. Therefore, it is possible that soluble BiP has no relationship with regulatory T cell frequencies but it may affect regulatory T cell function.

The aim of this chapter is to determine if soluble BiP has any effect on regulatory T cell function. Other heat shock proteins have been shown to affect regulatory T cell function. HSP60 (Zanin-Zhorov et al, 2006) and HSP70 (Wachstein et al, 2012) have been shown to enhance regulatory T cell function by enhancing regulatory T cell ability to suppress responder T cell proliferation and IFN-γ and TNF-α levels from the co-culture. In addition, HSP60 and HSP70 pre-treatment on regulatory T cells can induce enhanced IL-10 and TGF-β secretions, both cytokines are anti-inflammatory cytokines. To achieve the aim of this chapter, experiments were performed using cone bloods. Regulatory T cells and responder T cells were isolated using magnetic beads before co-culture experiments were performed. BiP pre-treatments on regulatory T cells were performed and washed away before being co-cultured with responder T cells. The readout of the suppression assays is responder T cell proliferation measured by tritiated thymidine incorporation and levels of cytokines, namely IFN-γ, TNF-α and IL-10, measured from culture supernatants using ELISA.

4.2 Objectives

The objectives of this chapter are as follows:-

- To optimise regulatory T cell and responder T cell isolation
- To optimise suppression assay of regulatory T cell
- To replicate the effect of HSP60 on regulatory T cell function as shown by Zanin-Zhorov et al (2006)
- To investigate the effect of BiP pre-treatment on regulatory T cell phenotype
- To investigate the effect of BiP pre-treatment on regulatory T cell function using suppression assay

4.3 Results

4.3.1 Regulatory and responder T cell isolation

Baecher-Allan et al (2001) showed that human regulatory T cells can be differentiated from activated T cells by looking at the intensity of CD25 expression. Regulatory T cells express high CD25 level with constitutive expression while activated T cells can express low levels CD25 transiently. This difference can be clearly seen in greater suppressive capacity of $CD25^{High}$ cells compared to $CD25^{Low}$ cells.

Regulatory and responder T cells were isolated from cone bloods. The isolation was performed by first diluting cone blood with 1X PBS up to 60 ml. Then, gradient density centrifugation was performed and PBMCs were obtained. Typically a cone blood can yield from 3 to 5 x 10^8 PBMCs. Then, a further isolation step to obtain regulatory and responder T cells was performed using magnetic beads. The isolation steps are illustrated in Figure 4.1. CD25 can be expressed not only by human regulatory T cells but can also be expressed by activated responder T cells.

As a control, the percentage of responder and regulatory T cells defined $CD4+CD25^{Low}CD127^{High}$ and $CD4+CD25^{High}CD127^{Low}$ populations were demonstrated in PBMC. Cells gated on the whole population (A) were further sub-gated on CD4 expression (B). As shown in Figure 4.2(C), 87.5% of the population are responder T cells and 7.7% are regulatory T cells. Therefore, further isolation step is needed to achieve higher purity for functional studies can be performed. The purity of responder T cells as defined $CD4+CD25^{Low}CD127^{High}$ cells (Figure 4.3) as the gating strategy was explained in this figure. Cells were gated on lymphocyte (A), then further sub-gated on CD4 expression (B) and responder T cells were defined as $CD25^{Low}CD127^{High}$ (C). The mean purity of responder T cells were 92.6% and the antibody staining was validated by comparing between antibody staining (red line) and isotype control staining (blue line) as depicted by histograms of CD4 (D), CD25 (E) and CD127 (F). Responder T cells isolated using magnetic beads using one MS column yielded 5 x 10^7 to 1 x 10^8 cells with a high purity of 93.2% of $CD4^+CD25^{Low}$ $CD127^{High}$ as shown in Figure 4.2.

Various additional markers have been suggested to further differentiate between regulatory T cells and other types of T cell subsets including activated T cells. One of the

surface markers suggested for this is CD127. CD127 is an alpha chain of IL-7 receptor and it has been suggested it may be absent from regulatory T cell subsets. Liu et al (2006) were looking at the relationship between CD127 expression, Foxp3 expression and the suppressive function of regulatory T cells. CD127 expression was found to be inversely correlated with Foxp3 expression and the suppressive function of human regulatory T cells. Therefore, regulatory T cells isolated for the experiments were checked for CD127 expression and regulatory T cells are defined as CD4+CD25^{High}CD127^{Low} cells (Figure 4.4).

Regulatory T cell purity depends on the number of MS columns used in the isolation. As shown in Figure 4.4, a similar gating strategy was used to define regulatory T cell population. Panel (A-C) show the purity of regulatory T cells was compared between isolation using 2 MS columns while panel (D-E) demonstrate the purity of regulatory T cells using 1 MS column. Similarly with responder T cells, antibody staining of regulatory T cells were compared to isotype control as shown in Panel (G-I). Regulatory T cells defined as CD4^{+}CD25^{High} CD127^{Low} population isolated using 2 MS columns achieved higher purity (95%) compared to regulatory T cells isolated using 1 MS column (83.5%). Regulatory T cell isolated with higher purity are important to these experiment since the main objective is to examine the effect of BiP treatment on regulatory T cells, not responder T cells.

To further confirm the purity of regulatory and responder T cells, intracellular Foxp3 staining was performed. Foxp3 protein is an important transcription factor which appears to function as a master regulator in the development and function of regulatory T cells. Figure 4.5 demonstrates Foxp3 staining of regulatory T cells (A) and responder T cells (C) compared to isotype control staining respectively (Panel B&D). Regulatory T cells consist of 82.3% of Foxp3+ cells while 11.4% of responder T cells are Foxp3+ cells. In addition, Corrigall et al (2004) demonstrated that soluble BiP can affect phenotype and function of CD14 monocytes. Hence, CD14 staining was performed to ensure that no CD14+ cells were present after isolation. Figure 4.6 shows CD14 staining performed on regulatory and responder T cells. Respective cell populations were gated based on forward versus side scatter (A,C&E) and further sub-gated according to CD14 expression (B,D&F). Monocytes were used as a positive control and CD14 is a lineage marker of monocytes. The table in Figure 4.5 shows a very small percentage of CD14+ cells can be found in regulatory (1.2%)

and responder T cells (0.78%) after isolation. It was viewed that the purity of the cell populations was sufficient to proceed with the co-culture for *in vitro* suppression assay.

4.3.2 Two hours of HSP60 pre-treatment maintains regulatory T cell phenotype yet enhances regulatory function.

It is important to reproduce the findings of Zanin-Zhorov et al (2006) to show that HSP60 can enhance regulatory T cell function. The set-up of this experiment was performed exactly as shown in their paper. In addition, Wachstein and colleagues (2012) showed that functional change in regulatory T cells by HSP70 pre-treatment can be correlated with the expansion of regulatory T cells as measured by Ki-67 staining, a marker associated with cell proliferation when incubated with HSP70. To confirm the previous observation, regulatory T cells were cultured with HSP60 and the surface markers were assessed and compared with regulatory T cells cultured in the absence of HSP60.

Figure 4.7 shows the effect of HSP60 pre-treatment on regulatory T cell phenotype as shown by CD4, CD25 and CD127 expression. However, HSP60 pre-treatment maintained and did not alter the regulatory T cell phenotype as shown in Panel the histogram of CD4, CD25 and CD127 by the overlapping lines between mock treated and HSP60-treated regulatory T cells (Panel B-D) in one donor and multiple donors as shown in Panel (E-G) even though CD127 expression was reduced in one donor. Next, the suppressive function of HSP60 pre-treated regulatory T cells were assessed using *in vitro* suppression assay (Figure 4.8). Regulatory T cells were co-cultured with responder T cell for 96 hours in the presence of plate-bound anti-CD3 antibodies and the proliferation and cytokines were measured. Responder T cell proliferation was measured by using tritiated thymidine incorporation and cytokines were measured using ELISA from culture supernatants. HSP60 pre-treatment of regulatory T cells reduced the proliferation of responder T cells (Panel A) and all supernatant cytokine levels (IFN-γ, TNF-α and IL-10) (Panel (C,E,G). However, the mean effect of HSP60 pre-treatment on regulatory T cells in multiple donors (Panel B,D,F,H) can be clearly seen in certain cytokine levels in the co-culture; not with the suppression of responder T cell proliferation. Co-culture of responder and regulatory T cells treated with HSP60 did not affect TNF-α and IL-10 but significantly reduced IFN-γ, a pro-inflammatory cytokine in agreement with Zanin-Zhorov et al (2006).

4.3.3 Optimisation of suppression assay

Using a similar experimental set-up as used for HSP60 pre-treatment, an *in vitro* suppression assay was performed to investigate the effect of soluble BiP on regulatory T cell function. 1 ng/ml of BiP was used in pre-treatment with regulatory T cells before being co-cultured with responder T cells in the presence of 5 µg/ml plate-bound anti-CD3 antibody for 96 hours. As shown in Figure 4.9, BiP pre-treatment on regulatory T cells did not affect responder T cell proliferation (Panel A), and slightly increased cytokine levels of IFN-γ, TNF-α and IL-10 from the co-culture compared to mock treated regulatory T cells. The ratio of responder to regulatory T cells used in this experiment was 1 to 1; therefore it is possible that the effect of BiP was masked by the fact that mock treated regulatory T cells achieved maximal suppressive capacity. Therefore, the suppression assay has to be optimised.

The first step of optimisation is to determine the kinetics of responder T cell proliferation to ensure that responder T cells reach their maximum proliferation in the presence of plate-bound anti-CD3 antibodies. To activate responder T cells and make them proliferate, TCR stimulation and co-stimulation is necessary to enable them to reach maximal proliferative capacity. TCR stimulation can be achieved by using anti-CD3 antibodies while co-stimulation can be provided in the form of anti-CD28 antibody. Therefore, the kinetics of responder T cell proliferation was determined using both anti-CD3 antibodies alone or anti-CD3/CD28 beads.

Figure 4.10 shows responder T cell proliferation with and without the presence of plate-bound anti-CD3 antibodies and anti-CD3/CD28 beads. Responder T cells stimulated in the presence of 5 µg/ml plate-bound anti-CD3 antibodies and 1 anti-CD3/CD28 bead to 5 cells achieved higher proliferation levels compared to proliferation in the presence of 1 µg/ml anti-CD3 antibody and 1 anti-CD3/CD28 beads to 10 cells (Panel A-B). Responder T cell proliferation peaked at Day 3 in the presence of all stimuli except for 1 µg/ml anti-CD3 antibody. Therefore, future suppression assays were performed and compared at Day 3.

To ensure optimal levels of suppression, the kinetics of the suppression assay was investigated. The suppression assay was performed on cone bloods for 3 or 4 days in the presence of both stimuli and the level of suppression compared to suppression assays between 3 and 4 days as shown by Figure 4.11. A higher percentage of suppression was

observed after 4 days in the co-culture compared to 3 days in the presence of both stimuli either in one (Panel A,B,D,E) and multiple donors (Panel C&F). Therefore, suppression assays will be performed in the presence of 5 μg/ml anti-CD3 antibody and 1 anti-CD3/CD28 bead to 5 cells for 4 days.

The last step for optimisation of the suppression assay was to find a sub-optimum level of suppression in the presence of anti-CD3 antibodies and anti-CD3/CD28 beads so that any enhanced suppression mediated by BiP pre-treatment can be seen as co-culture of responder and regulatory T cells in a ratio of 1 to 1 achieved very high levels of suppression. Next, a titration of regulatory T cells to responder T cells in co-culture was performed to find a sub-optimal level of suppression. As shown in Figure 4.12, co-culture of responder and regulatory T cells in a ratio of 1 to 1 achieved 89% (anti-CD3 antibody, Panel B) and 80% (anti-CD3/CD28 beads, Panel C) suppression compared to 55% (both stimuli, Panel B&C) suppression when responder and regulatory T cells were co-cultured in ratio of 1 to 0.3. Therefore, the ratio that will be used for further suppression assays will be 1 responder T cell to 0.3 regulatory T cell.

In conclusion, the suppression assay has been optimised. Future suppression assays will be performed for 4 days in the presence of 5 μg/ml anti-CD3 antibodies or 1 anti-CD3/CD28 bead to 5 cells in the ratio of 1 responder T cell to 0.3 regulatory T cells.

To further confirm that the enhanced suppression observed at 4 days is not due to an increase in dying cells, PI staining was performed as shown in Figure 4.13. Cells with positive PI staining can be acquired by flow cytometry with a fluorescence excitation maximum of 535 nm and an emission maximum of 617 nm. Comparison of PI positive cells between stimulated responder T cells (9% for anti-CD3 antibody and 12% for anti-CD3/CD28 beads) and a co-culture of responder to regulatory T cells (10% for anti-CD3 antibody and 10% for anti-CD3/CD28 beads) was performed and shown in multiple donors (Panel I-J). This data reveal a similar percentage of dead cells between stimulated responder T cells and the co-culture of responder and regulatory T cells in both stimuli.

4.3.4 Two hours of BiP pre-treatment does not enhance regulatory T cell function in the presence of anti-CD3 antibodies or anti-CD3/CD28 beads

Various doses of BiP ranging from 0.1 ng/ml to 1 µg/ml were incubated with regulatory T cells before suppression assays were performed in the presence of both stimuli (Panel A-B). BiP pre-treatment of 0.1 until 10 ng/ml showed a dose-dependent suppression in the presence of anti-CD3 antibody (Panel A) while no enhanced suppression observed with BiP pre-treatment in the presence of anti-CD3/CD28 beads (Panel B). Data from Panel A indicated that 10 ng/ml is the optimum dose to be used in future experiments concerning BiP pre-treatment of regulatory T cells as shown in Figure 4.14 because these achieved the highest reduction of responder T cell proliferation. After that, 10 ng/ml of BiP was used for BiP pre-treatment on regulatory T cells before being co-cultured with responder T cells for 4 days as shown in Figure 4.15.

BiP pre-treatment of regulatory T cells in suppression assays using anti-CD3 antibodies had a variable effect on the level of suppression. Data from one donor showed that 10 ng/ml of BiP pre-treatment can reduce the proliferation of responder T cells in the presence of anti-CD3 antibody (blue bars, Panel A) but not anti-CD3/CD28 beads (Yellow bars, Panel C). To control for any carry over effect of BiP pre-treatment on regulatory T cells, responder T cells were cultured with BiP in the presence of both stimuli (1st bar from right) (Panel A&C).

However, the mean effect of BiP pre-treatment in multiple donors showed different effect. In the presence of anti-CD3 antibody (Panel B), 4 donors achieved higher level of suppressions when comparing with BiP treated regulatory T cells and mock treated regulatory T cells while another 4 donors obtained a lower level of suppression compared to mock treated regulatory T cells. This variability brings no significant change in term of suppression level when compared with mock treated regulatory T cells and BiP-treated regulatory T cells ($p=1$, $n=8$). On the other hand, the effect of BiP pre-treatment in suppression assays using anti-CD3/CD28 beads were measured from 6 donors. 2 donors were excluded because of high variability in suppression. In 5 out of 6 donors, BiP pre-treatment on regulatory T cells slightly increased the suppression level but the difference between mock treated and BiP treated regulatory T cells in terms of the percentage level of suppression is not statistically significant ($p=0.44$, $n=6$) (Panel D).

To further confirm that BiP pre-treatment has no effect on regulatory T cell function, supernatant cytokine levels of IFN-γ, TNF-α and IL-10 were measured as shown in Figure 4.16. In the presence of anti-CD3 antibodies or anti-CD3/CD28 beads, no significant difference was observed between cytokine levels of the co-culture of mock treated or BiP treated regulatory T cells and responder T cells of IFN-γ (Panel A&D), TNF-α (Panel B&E) and IL-10 (Panel C&F).

4.3.5 Two hours of BiP pre-treatment maintains regulatory T cell phenotype

To confirm that BiP pre-treatment has no effect on regulatory T cell phenotype, the surface marker expression of CD4, CD25 and CD127 were assessed and compared between mock treated and BiP treated regulatory T cells. Phenotypic changes in regulatory T cells were assessed using flow cytometry. Surface markers assessed were CD4, CD25 and CD127, and the markers that were used to validate regulatory T cell staining as regulatory T cells are defined as CD4+CD25^{High}CD127^{Low} population. Figure 4.17 shows histograms of CD4, CD25 and CD127 expression on regulatory T cells. The red line indicates regulatory T cells cultured in the absence of BiP while the blue, orange and green lines represents regulatory T cells cultured in the presence of various doses of BiP (0.1,1,10 ng/ml) for 24 hours respectively (Panel B-D). Clearly there is no difference as all lines overlap and this figure is a representative experiment in 4 donors as no changes were observed. In addition, the mean fluorescence intensity (MFI) of each marker was examined in multiple donors (Panel E-G). Regulatory T cells cultured in the presence of various doses of BiP demonstrated variable effect of MFI of CD4, CD25 and CD127 between mock treated and BiP treated regulatory T cells. This result further confirms the earlier finding that BiP pre-treatment on regulatory T cells has no effect on responder T cell proliferation and supernatant cytokines from the suppression assay. In conclusion, 2 hours of BiP pre-treatment does not change the phenotype and function of regulatory T cells.

4.3.6 BiP can reduce responder T cell proliferation in the presence of anti-CD3/CD28 beads but not the cytokine levels from the culture

To control for any effect of left over BiP not removed during the washing step of regulatory T cells on responder T cells, responder T cells were cultured with BiP in the presence of both stimuli as shown in Figure 4.15 (Panel A&B). Interestingly, BiP can significantly reduce proliferation of responder T cells after 4 days in the culture in the

presence of anti-CD3/CD28 beads in multiple donors as shown in Figure 4.18 (B) (p=0.0078, n=8), but not in the presence of anti-CD3 antibodies only (Panel A) (p=0.94, n=8). BiP effect on responder T cells was further investigated on the cytokine profile secreted from responder T cells (Figure 4.19). IFN-γ, TNF-α and IL-10 measured from the culture of responder T cells with BiP showed variability hence no significant effect was observed when compared to responder T cells cultured without BiP either in the presence of anti-CD3 antibody (Panel A-C) or anti-CD3/CD28 beads (D-F).

4.3.7 Optimisation of suppression assay in the presence of monocytes

The use of anti-CD3 antibodies and anti-CD3/CD28 beads to activate responder T cells in co-culture with regulatory T cells does not mimic the physiological situation. Therefore, it is possible that BiP may affect regulatory T cell function when co-cultured with APC. To perform a co-culture of monocytes, responder and regulatory T cells, isolation of the cell populations was performed using magnetic beads as shown in Figure 4.20. The purity of monocytes isolated from magnetic beads was assessed using anti-CD14 staining as the gating strategy was shown in Figure 4.20(A-B). CD14 is a lineage marker of monocytes and the antibody staining of CD14 was validated when compared with isotype staining control as demonstrated in Figure 4.21(C). Monocytes isolated using magnetic beads achieved high purity of 73.6% of CD14+ cells in multiple donors (n=5) as demonstrated in Figure 4.20.

Before the suppression assay can be performed, optimisation of co-cultures has to be performed. Responder T cells cultured with monocytes achieved high proliferation even in the absence of anti-CD3 antibody. However, in the presence of anti-CD3 antibody, co-culture of responder T cells with monocytes recorded higher proliferation (30639 CPM) compared to co-culture of responder T cells with monocytes in the absence of anti-CD3 antibody (15962 CPM). Responder T cells cultured with monocytes in the presence of anti-CD3 antibodies peak at Day 2 as illustrated in Figure 4.22; by contrast to 3 days of responder T cell culture in the presence of anti-CD3 antibodies or anti-CD3/CD28 beads (Figure 4.10).

4.3.8 Two hours of BiP pre-treatment on regulatory T cells does not enhance their function even in the presence of monocytes

Corrigall et al (2004) demonstrated that BiP can affect monocyte function; therefore the effect of BiP on regulatory T cells may be facilitated via interaction with monocytes. Walter et al (2013) reported that activated human monocytes can interact with regulatory T cells and cause an increase of the frequency of IL-17 secreting regulatory T cells. However, these regulatory T cells had enhanced capacity to suppress responder T cell proliferation and IL-17 secretion from the co-culture. This clearly shows that cross-talk between monocytes and regulatory T cells can happen. Therefore, BiP pre-treatment was performed on regulatory T cells and co-cultured with monocytes before responder T cells were added 24 hours later to examine the suppressive capacity of responder T cell.

Figure 4.23(A) shows the proliferation measured from responder T cells proliferation from one donor. The proliferation of responder T cells cultured in the presence of monocytes and anti-CD3 antibody (3rd bar from right) was reduced in the presence of mock-treated regulatory T cells (2nd bar from right). However, responder T cells achieved higher proliferation level in the presence of BiP treated regulatory T cells (1st bar from right) compared to mock treated regulatory T cells. The similar effect can be observed with IFN-γ levels measured from the co-culture (Panel B). However, no change was observed with TNF-α and IL-10 levels measured from the co-culture with BiP treated regulatory T cells compared to mock treated regulatory T cells (Panel C-D). The data from multiple donors showed that 2 hours of BiP pre-treatment does not enhance regulatory T cell function as measured by proliferation of responder T cells and supernatant cytokines from the co-culture.

4.3.9 Two hours of BiP pre-treatment on monocytes does not enhance regulatory T cell in co-culture but reduces proliferation of responder T cells

Two hours of BiP pre-treatment was performed on monocytes before being co-cultured with regulatory T cells. Responder T cells were added into the culture 24 hours later as shown in Figure 4.24 and the proliferation of one donor was shown in Panel A. The co-culture of regulatory, responder and mock treated monocytes shows the reduction of proliferation (2nd bar from right) compared to the co-culture of responder T cells and mock-treated monocytes (4th bar from right) indicating that regulatory T cells can suppress the

proliferation of responder T cells. Similarly, regulatory T cells can suppress responder T cells in the presence of BiP-treated monocytes (comparing between 3rd bar and 1st bar from right). The proliferation data were converted into percentage of suppression of multiple donors as shown in Panel (B). The effect of BiP pre-treatment on monocytes on regulatory T cell suppressive capacity is variable as 3 of 5 donors showed the percentage of suppression reduced upon BiP pre-treatment of monocytes, while the remaining donors showed enhanced suppression therefore the indirect effect of BiP on regulatory T cells via monocytes is not statistically significant (p=0.31, n=5).

In addition, supernatant cytokines were measured from the co-culture. Supernatant IFN-γ measured from the co-culture showed similar trend with the proliferation data (Panel C&D) with 3 donors showed increased IFN-γ levels with BiP treated monocytes in the co-culture compared to mock treated monocytes whilst the remaining donors showed otherwise (p=0.62). The variable effect of BiP pre-treatment on monocytes on the suppressive capacity of regulatory T cells and IFN-γ levels from the co-culture was replicated in TNF-α and IL-10 levels. 4 of 5 donors showed higher levels of TNF-α and IL-10 levels in the co-culture with BiP-treated monocytes compared to mock treated monocytes as shown in Panel (E-G). Hence, no significant difference was observed between TNF-α (p=1) and IL-10 (p=0.32) levels between BiP treated and mock treated monocytes.

However, Figure 4.24(A) suggests that BiP-treated monocytes can reduce the proliferation of responder T cells without the addition of regulatory T cells (comparing between 4th bar and 3rd bar from right). Therefore, the effect of BiP treated monocytes on responder T cells was examined. As shown in Figure 4.25(A), BiP treated monocytes can reduce the proliferation of responder T cells compared to mock treated monocytes in all 5 donors even though this is not statistically significant (p=0.06). The effect of BiP indirectly on responder T cells is limited to responder T cell proliferation only as variable effect was observed with the supernatant cytokine levels of IFN-γ (p=0.82), TNF-α (p=0.62) and IL-10 (p=0.18) measured from the co-culture as demonstrated in Figure 4.25(B-D).

4.3.10 Regulatory T cells must be cultured in the presence of IL-2

In this chapter, it has been shown that BiP can affect responder T cell proliferation directly in the presence of anti-CD3/CD28 beads (Figure 4.18) or indirectly via BiP-treated

monocytes (Figure 4.25). This shows that BiP can affect responder T cells by reducing their proliferation when cultured with BiP in a period longer than 2 hours. Therefore, the effect of BiP on regulatory T cells could be visible if incubated for more than 2 hours. Since the effect of BiP on responder T cell proliferation can be seen after 4 days in culture, regulatory T cells were cultured for 4 days. It has been established that IL-2 is an important survival factor for regulatory T cells and shown in Figure 4.26 where regulatory T cells cultured in the absence of IL-2 had higher percentage of death cells (20.1%) (Panel C) compared to regulatory T cells cultured in the presence of IL-2 (6.45%) (Panel D). This effect is clear in multiple donors as shown in Panel (E) where regulatory T cells cultured in the absence of IL-2 had an approximately 6-fold increase in dying cells (35% propidium iodide+ cells) compared to regulatory T cells cultured in the presence of IL-2 (6% propidium iodide+ cells). Therefore regulatory T cells were cultured with or without BiP in the presence of IL-2 to investigate the effect of BiP on regulatory T cells over 4 days.

4.3.11. The kinetics of BiP effect on regulatory T cells

To investigate the effect of BiP on regulatory T cells, regulatory T cells were cultured in the presence of IL-2 with or without 10 ng/ml of BiP. As BiP can reduce responder T cell proliferation in the presence of anti-CD3/CD28 beads after 4 days in culture as shown in Figure 4.18 while BiP can indirectly reduce responder T cell proliferation via BiP-treated monocytes after 2 days in culture as demonstrated in Figure 4.25. Therefore, regulatory T cells will be cultured with BiP for 3 time periods; 2, 4 and 6 days (Panel A-F). Regulatory T cells cultured with or without BiP in the presence of IL-2 showed similar levels of dying cells on Day 2 (4.4%) and 4 (5.4%) as shown in Figure 4.27(G). However, the percentage of PI+ cells in regulatory T cells cultured without BiP increased 2 fold at Day 6 (10.7%) compared to Day 4 (5.4%). Regulatory T cells cultured with BiP always recorded a lower percentage of PI+ cells compared to regulatory T cells cultured without BiP throughout 3 different time points.

The effect of BiP on regulatory T cell phenotype was investigated by looking at the expression of Foxp3 and CD25, two important molecules used in the identification of human regulatory T cells. Regulatory T cells used in this experiment were isolated using 1 MS column as fresh bloods were used. Therefore, the purity of regulatory T cells from most donors were lower compared to regulatory T cells isolated using 2 MS columns as demonstrated in Figure 4.4. Foxp3 versus CD25 expression of regulatory T cells cultured

with or without BiP gated from the lymphocyte gate of one donor was demonstrated in Figure 4.28(A-F) throughout 3 different time points. The effect of BiP on regulatory T cell frequency varies at 3 different time points. The histogram in Panel (G-H) show the histogram of CD25 and Foxp3 of regulatory T cell cultured with (blue line) or without BiP (red line). Foxp3 and CD25 expression were enhanced in the presence of BiP as shown by the MFI of Foxp3 and CD25 of regulatory T cells cultured with BiP which is higher compared to regulatory T cells cultured without BiP (Foxp3: 224 versus 168, CD25: 586 versus 557). The comparison between Foxp3 and CD25 antibody staining and isotype control staining was performed and shown in Panel (I-J) to confirm that the antibody staining is working.

The cumulative data of BiP effect on regulatory T cells can be observed in Panel (K-M). The frequency of the Foxp3+CD25+ population was not affected on Day 2 and 4 with BiP in the culture while the frequency of Foxp3+CD25+ population was reduced on Day 6 (shown in Panel K). Both Foxp3 and CD25 expression measured by their MFI expression were modulated by BiP treatment on regulatory T cells (Panel L-M) compared to the frequency of the Foxp3+CD25+ population. Foxp3 and CD25 expression showed a trend of higher expression in regulatory T cells cultured with BiP compared to regulatory T cells cultured without BiP at Day 2 and 4 although the differences are statistically insignificant (Foxp3: Day 2 & 4, p=0.8, CD25: Day 2, p=0.8, Day 4, p=0.6) . However, the mean MFI of Foxp3 and CD25 of regulatory T cells were the highest at Day 4 and went down at Day 6 perhaps because of the increase in cell death as shown in the previous figure.

In addition to investigating the phenotype of regulatory T cells, the cytokines-associated with regulatory T cells were measured using ELISA, namely IL-10 and TGF-β1. Latent TGF-β1 was measured from the culture because it does not need to be activated. Activation of TGF-β1 using a neutralisation step introduced a great variability as indicated by the standard error. Therefore, TGF-β measured from the culture represents latent TGF-β1 measurement. Figure 4.29(A) demonstrates that IL-10 produced by regulatory T cells cultured without BiP in the presence of IL-2 was slightly higher compared to regulatory T cells cultured with BiP (Day 2 & 6, p=0.3, Day 4, p=0.6). However, the opposite effect was observed on TGF-β produced in the culture (Panel B). Regulatory T cells cultured with BiP showed a trend toward higher TGF-β secretion compared to regulatory T cells cultured without BiP at all Day 2 (p=0.6) and 6 (p=1.0) although the differences are statistically

insignificant. Both IL-10 and TGF-β secretion were the highest at Day 6 therefore Day 6 could be used to investigate cytokine production in regulatory T cell in cultures. However, a literature search revealed that TGF-β can be secreted by macrophages upon phagocytosis of apoptotic cells (Barker et al, 2002). Therefore, further investigation of the BiP effect on regulatory T cells was performed at Day 4 instead of Day 6 as more cells started to die and apoptotic cells may induce TGF-β secretion which may interfere with secretion from regulatory T cells cultured with BiP.

4.3.12 Four day treatment of BiP on regulatory T cells does not change Foxp3 and CD25 expression and IL-10 and TGF-β secretion

Phenotypic analysis of CD25 and Foxp3 was further performed on 13 donors after 4 day treatment with BiP as shown in Figure 4.30. The frequency of Foxp3+CD25+ cells was slightly increased by 4 day BiP treatment as demonstrated in Panel (A-B). However, the histogram of Foxp3 (Panel C) shows that Foxp3 expression regulatory T cells cultured with BiP (blue) was enhanced compared to regulatory T cells cultured without BiP (red line) and indicated by the MFI value but CD25 expression was lower in BiP-treated regulatory T cells compared to mock-treated regulatory T cells (shown in Panel (D)). However, the cumulative effect of 4 day BiP treatment on regulatory T cells did not change Foxp3+CD25+ frequency ($p=0.44$) and CD25 MFI ($p=1$) in multiple donors as demonstrated in Panel (E-F). In contrast, 4 day BiP treatment on regulatory T cells can increase Foxp3 expression as measured by Foxp3 MFI (310) compared to regulatory T cells cultured without BiP (310) although the increase is statistically insignificant ($p=0.06$) as illustrated in Panel (G).

Panel A demonstrate regulatory T cells secrete lower levels of IL-10 in the culture with BiP (5 pg/ml) compared to regulatory T cells cultured with BiP (9 pg/ml) ($p=0.8$, $n=15$). In contrast, TGF-β secretion in regulatory T cells was higher when cultured with BiP (365 pg/ml) compared to regulatory T cells cultured without BiP (279 pg/ml) (Panel B). This is a similar trend with Foxp3 expression as the increase in TGF-β secretion in regulatory T cells cultured with BiP is not significant compared to regulatory T cells cultured without BiP ($p=0.09$, $n=15$).

In conclusion, BiP may affect the regulatory T cell phenotype. Although the differences in Foxp3 expression and TGF-β secretion are statistically insignificant, BiP

treatment of regulatory T cells showed higher Foxp3 expression and TGF-β secretion. As demonstrated by HSP60 data, HSP60 pre-treatment can affect regulatory T cell function to reduce IFN-γ although no phenotypic change was observed with HSP60 pre-treatment. Therefore, it is important to investigate whether 4 days of BiP treatment may induce changes to regulatory T cell function by using *in vitro* suppression assay.

4.3.13 Four day treatment of BiP on regulatory T cells does not enhance their regulatory function

As regulatory T cells cultured with BiP for 4 days can enhance Foxp3 expression and induce TGF-β secretion, it is therefore important to assess whether these changes can contribute to enhanced suppressive function of regulatory T cells. The suppressive function of regulatory T cells must be investigated after being cultured for 4 days in the presence of IL-2. Figure 4.32 (A) shows that regulatory T cells can still suppress the proliferation of responder T cells in the presence of anti-CD3/CD28 beads (comparing between 3rd bar and 2nd bar from right). The addition of BiP-treated regulatory T cells can further enhance the suppression of the proliferation of autologous responder T cells (comparing between 2nd bar and 1st bar from right). However, the effect was variable as 3 of 6 donors showed enhanced suppression with BiP-treated regulatory T cells in the co-culture, while the remaining donors showed opposite effect; therefore the difference is not statistically significant (p=0.6875).

Supernatant cytokine levels of IFN-γ, TNF-α and IL-10 were measured from the co-culture. BiP-treated regulatory T cells can reduce the IFN-γ in the co-culture compared to mock treated regulatory T cells (Panel C) whilst TNF-α (Panel E) and IL-10 levels (Panel G) were higher in the co-culture of BiP-treated regulatory T cells and autologous responder T cells compared to mock treated regulatory T cells in the co-culture. However, the effect of 4 day BiP treatment is variable in multiple donors of supernatant cytokine levels of IFN-γ (Panel D), TNF-α (Panel F) and IL-10 (Panel H) therefore the difference is not statistically significant (all cytokines, p=0.62, n=6).

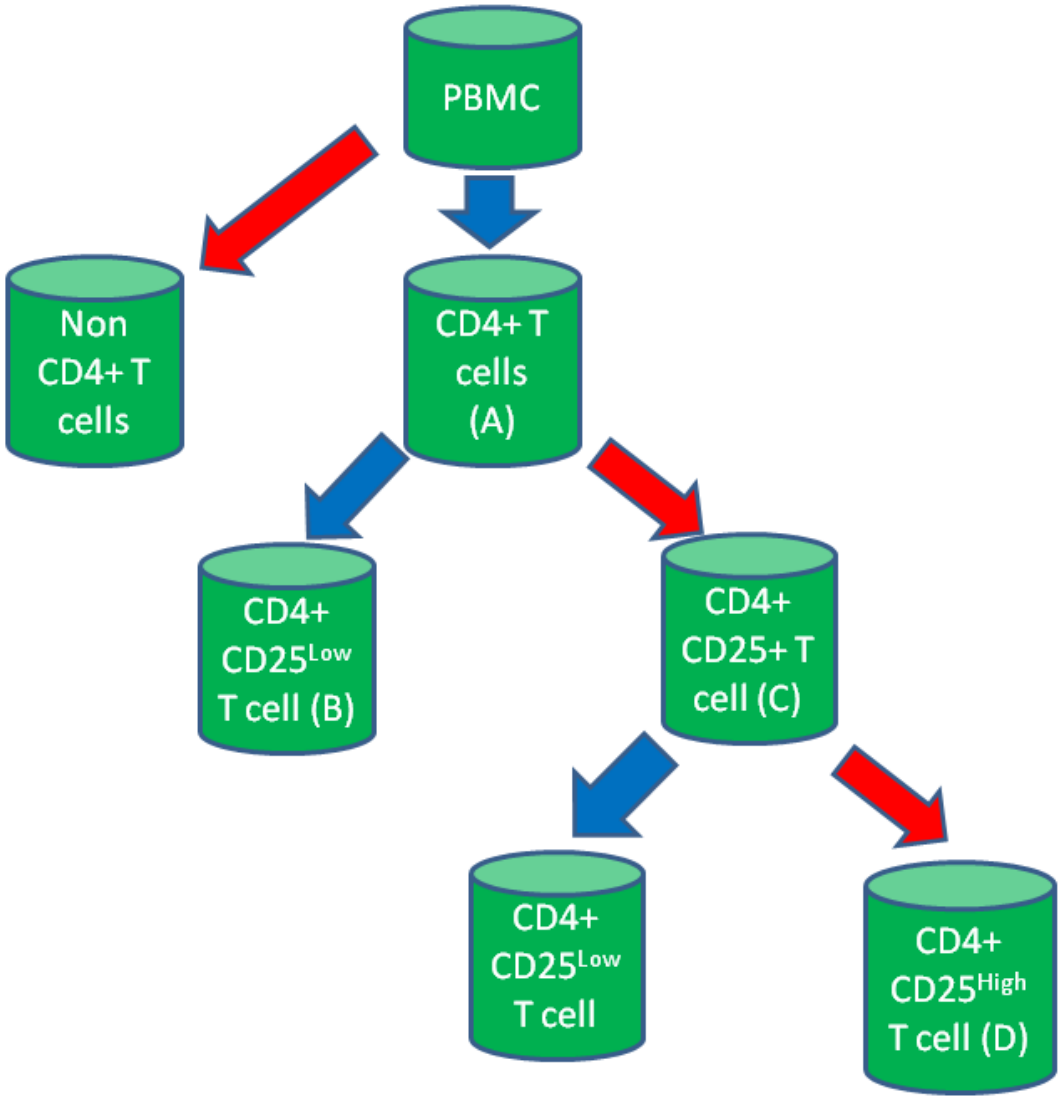


Figure 4.1. A schematic diagram showing how regulatory and responder T cells were isolated using Human CD4+CD25+ Regulatory T cell Isolation Kit (Miltenyi Biotech). PBMCs were obtained using density gradient centrifugation using Lymphoprep. After that, PBMCs were incubated with CD4+ T cell Biotin-Antibody Cocktail and Anti-Biotin Microbeads. Then, CD4+ T cells were isolated using LD column. CD4+ T cells (A) were collected as flow through of LD column as indicated by the blue arrow. After that, CD4+ T cells were incubated with CD25 antibodies and isolated using MS column. Responder T cells, defined as CD4+ $CD25^{Low}$ population, were collected as flow through of MS column (B) while regulatory T cells can be collected by either washing away cells bound to the first MS column (C) or collected by washing away cells bound to the second MS column (D). Both populations were stained with anti-human CD4, CD25 and CD127 antibodies and the purity was assessed using flow cytometry. The red arrows indicate positive selection while blue arrows indicate negative selection.

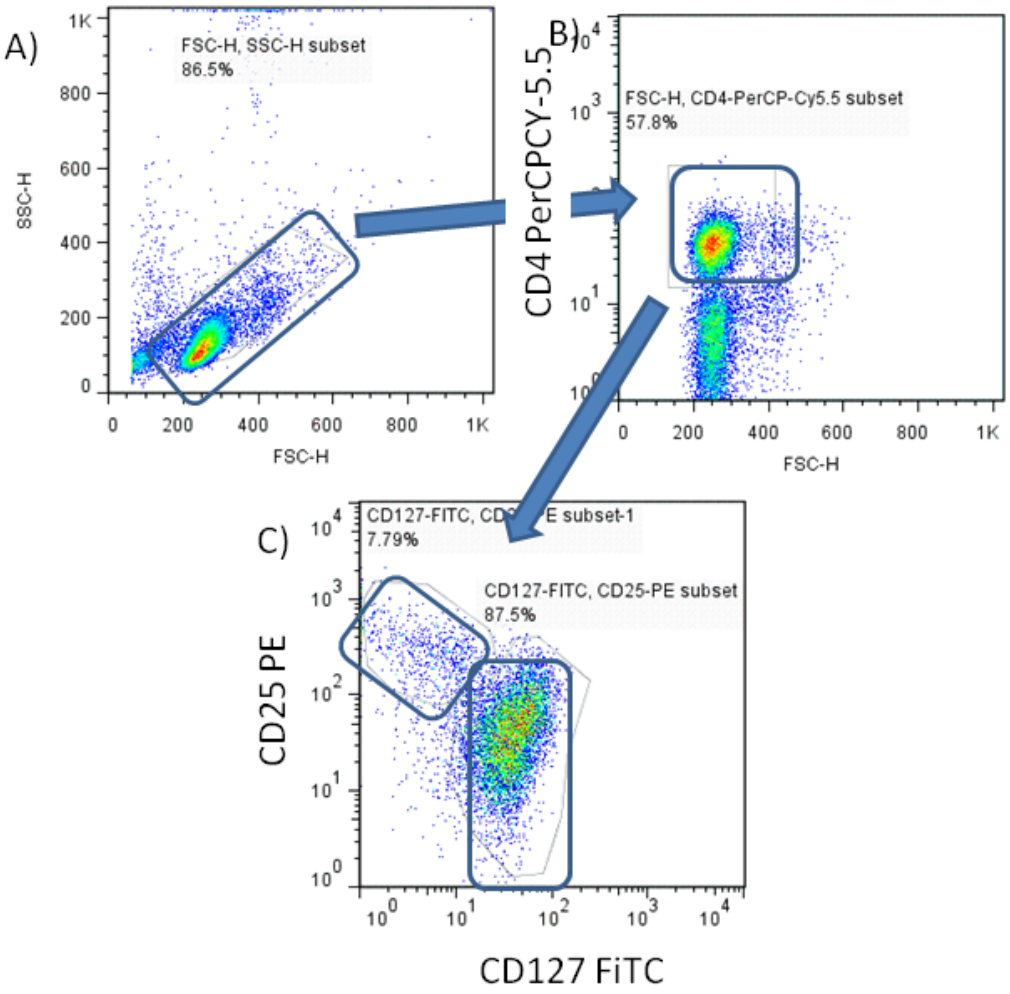
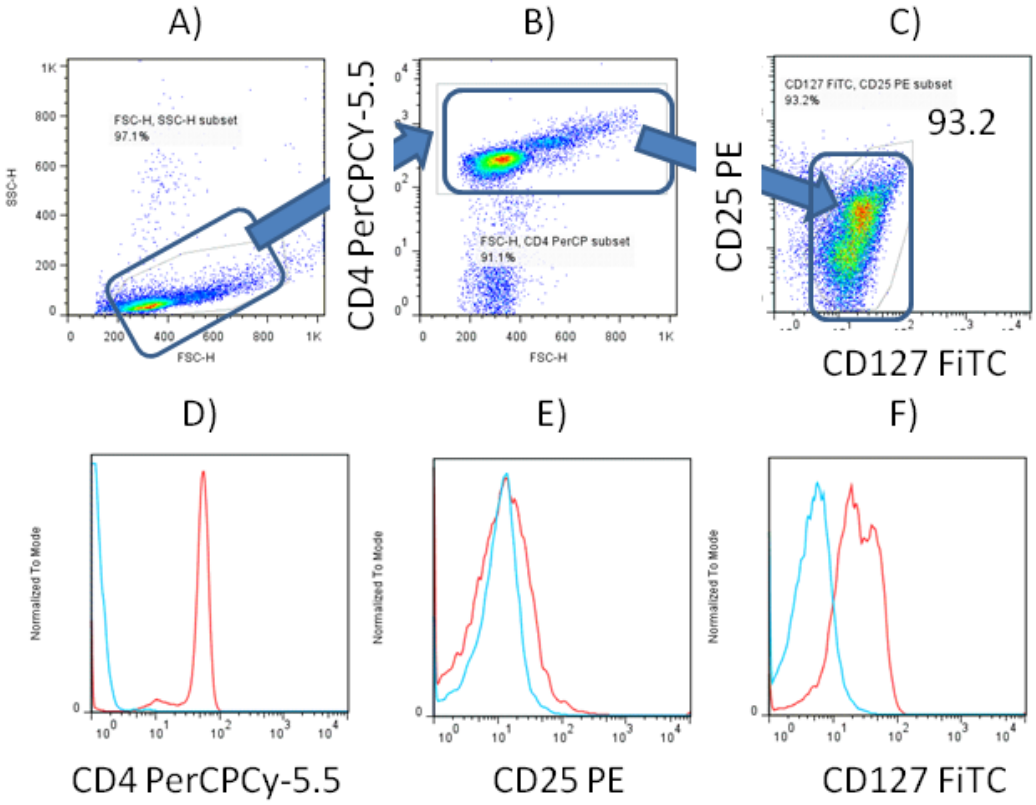
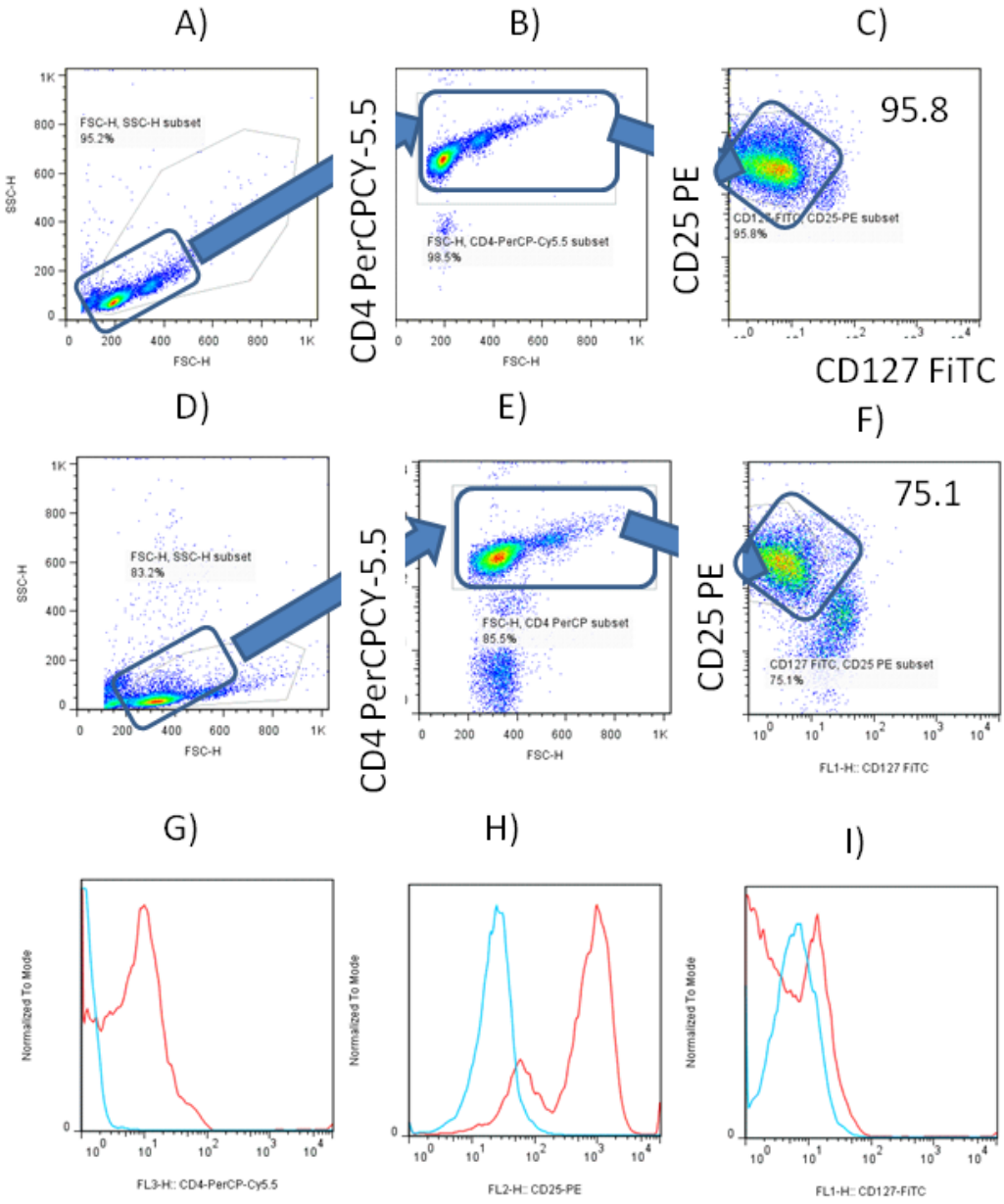


Figure 4.2 Regulatory and responder T cells need to be further isolated using MACS to achieve higher purity before any functional experiment can be performed. This experiment was performed on cone bloods and the isolation was performed by first isolating PBMC using the gradient density centrifugation method. After that, cells were stained with human anti-CD4, anti-CD25 and anti-CD127 antibodies. Cells were acquired using FACS Calibur and data was analysed using FlowJo. Panel (A) shows Forward v Side Scatter and the cells were gated on lymphocyte. Then, the cells were further gated based on CD4 expression as shown in (B). Next, the cells were checked for CD25 against CD127 expression and gated based on regulatory T cell ($CD25^{High}CD127^{Low}$) and responder T cell ($CD25^{Low}$ $CD127^{High}$) populations (C).



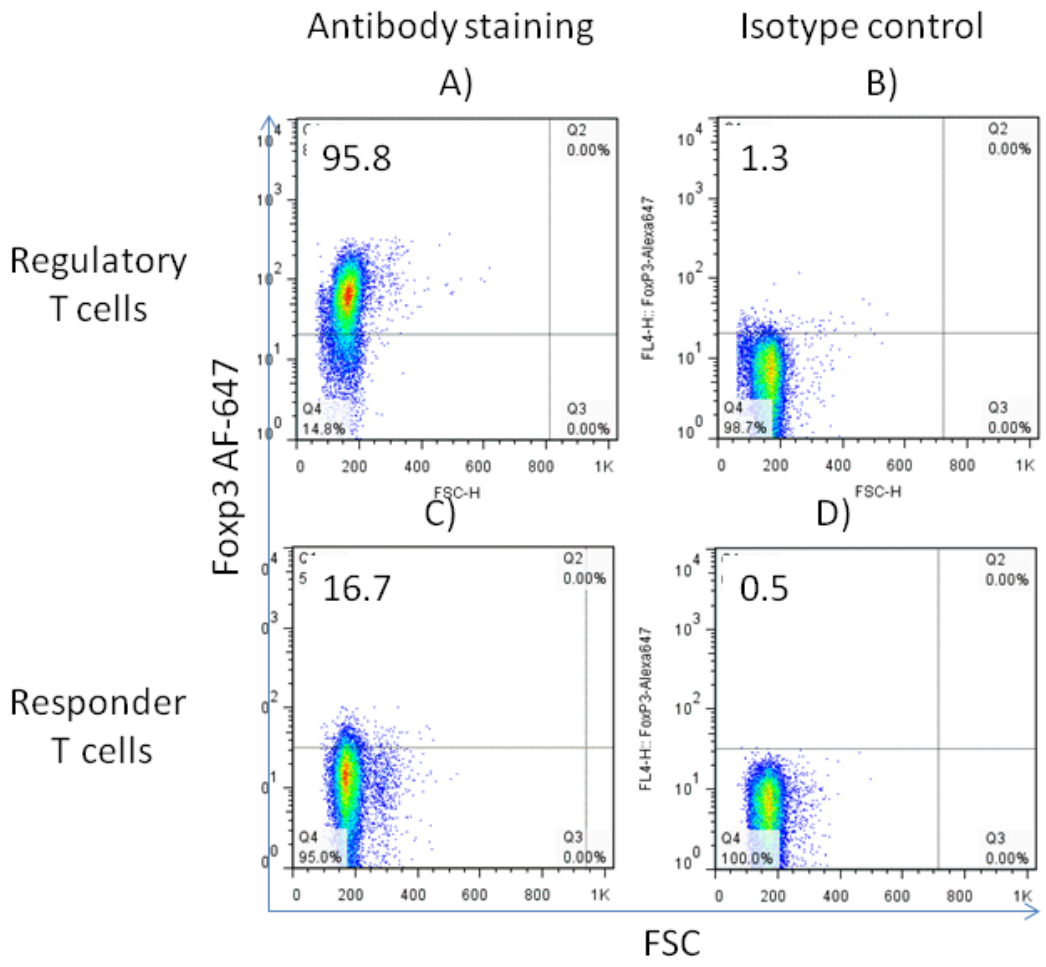
Responder T cell	CD4+CD25^{Low}CD127^{High} (%±SD)
1 MS Column (n=33)	92.6±1.5

Figure 4.3 High purity of responder T cell population after isolation using Human CD4$^+$CD25$^+$ Regulatory T cell isolation kit. This experiment was performed on cone bloods and the isolation was performed by first isolating PBMC using the gradient density centrifugation method. Then, the isolation was performed according to the manufacturer's instructions. After that, cells were stained with human anti-CD4, anti-CD25 and anti-CD127 antibodies. Cells were acquired using FACS Calibur and data was analysed using FlowJo. Panel (A) shows Forward v Side Scatter and the cells were gated on lymphocyte. Then, the cells were further gated based on CD4 expression as shown in (B). Next, the cells were checked for CD25 against CD127 expression and gated based on CD25^{Low} CD127^{High} population (C). Panel (D-F) show a histogram comparing antibody staining (red line) against isotype control (blue line) for CD4 (D), CD25 (E) and CD127(F). The table demonstrates the mean purity of responder T cells ±standard deviation after passage over 1 MS column.



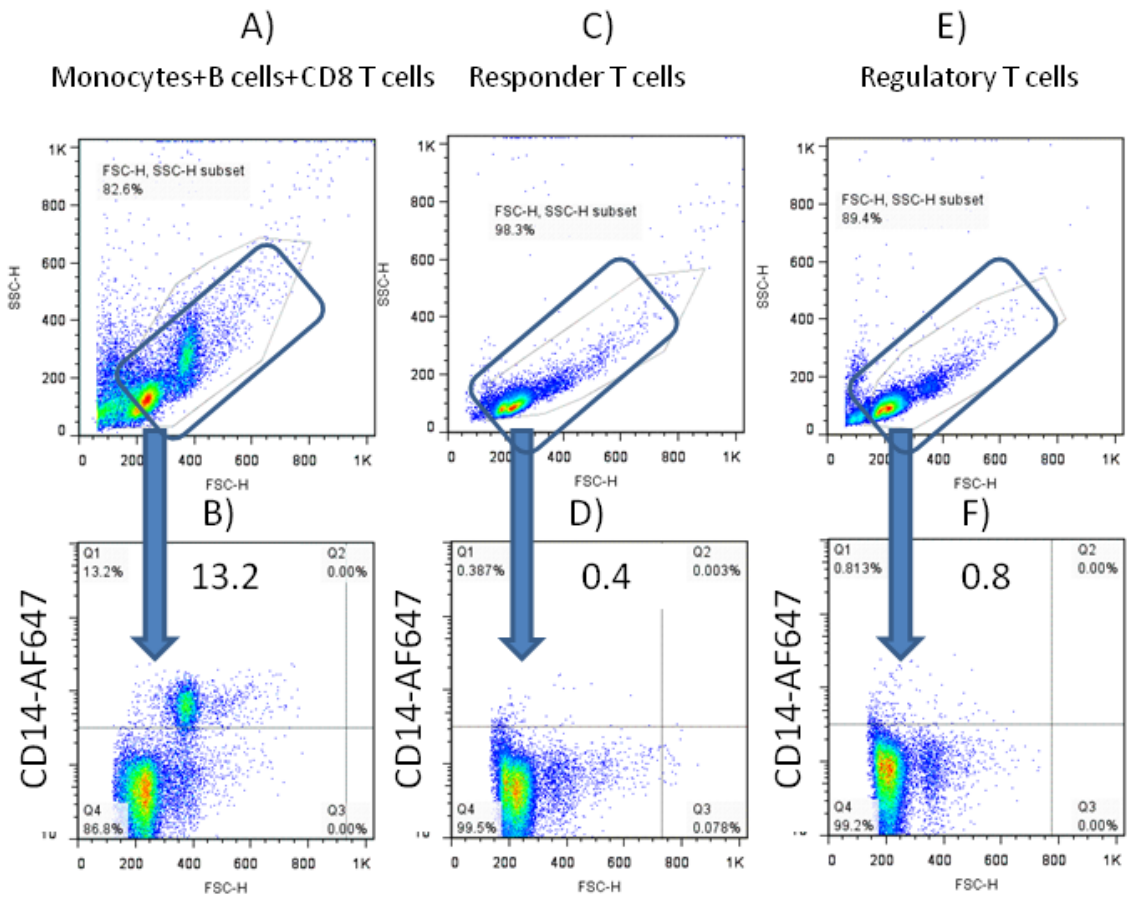
Regulatory T cell	CD4+CD25^{High}CD127^{Low} (%±SD)
1 MS column (n=5)	83.52±8.1
2 MS column (n=28)	95±2.2

Figure 4.4 Higher purity of regulatory T cell isolated using 2 MS columns compared to 1 MS column. This experiment was performed on cone bloods and the isolation was performed by first isolating PBMC using the gradient density centrifugation method. After that, cells were stained with human anti-CD4, anti-CD25 and anti-CD127 antibodies. Cells were acquired using FACS Calibur and data was analysed using FLowJo. Panel (A-C) show regulatory T cell isolated using 2 MS columns while Panel (D-F) show regulatory T cell isolated using 1 MS column. Cells were first gated on lymphoycte gate (A&D) and further sub-gated on CD4 expression as shown in (B&E). Next, the cells were examined for CD25 against CD127 expression and gated based on $CD25^{High}CD127^{Low}$ population (C&F). Panel (G-I) show a histogram comparing antibody staining (red line) against isotype control (blue line) for CD4 (G), CD25 (H) and CD127(I). The table shows the mean purity of regulatory T cells between 1 and 2 MS columns±standard deviation.



Cell types	Foxp3+ (%±SD)
Responder T cell (n=2)	11.4
Regulatory T cell (n=4)	82.3±3

Figure 4.5 The majority of regulatory T cells are Foxp3+ cells. This experiment was performed on cone blood and the isolation was performed as described in Figure 4.1. Intracellular staining of Foxp3 was performed using the intracellular staining kit from BioLegend. Cells were acquired using FACS Calibur and data was analysed using FLowJo. Panel (A) shows Foxp3 expression of regulatory T cells isolated using the 2 MS column compared with isotype control (B) while Panel (C) demonstrates Foxp3 expression for responder T cells compared to isotype control (D). The table demonstrates the mean frequency of Foxp3+ cells in regulatory and responder T cells ±standard deviation.



Cell types (n=3)	CD14+ (%±SD)
Monocytes+B cells	18.13±5.4
Responder T cells	0.78±0.4
Regulatory T cells	1.2±0.4

Figure 4.6 Low contamination of CD14+ monocytes after responder and regulatory T cells isolation. This experiment was performed on cone bloods and the isolation was performed as shown in Figure 4.1. After that, cells were stained with human anti-CD14 antibody. Cells were acquired using FACS Calibur and data was analysed using FLowJo. Figure (A) shows forward v side scatter of monocytes and B cells and CD14 expression while the remaining figures show CD14 expression on responder T cells (C&D) and regulatory T cells (E&F). The table demonstrates the mean frequency of CD14% in different cell populations±standard deviation.

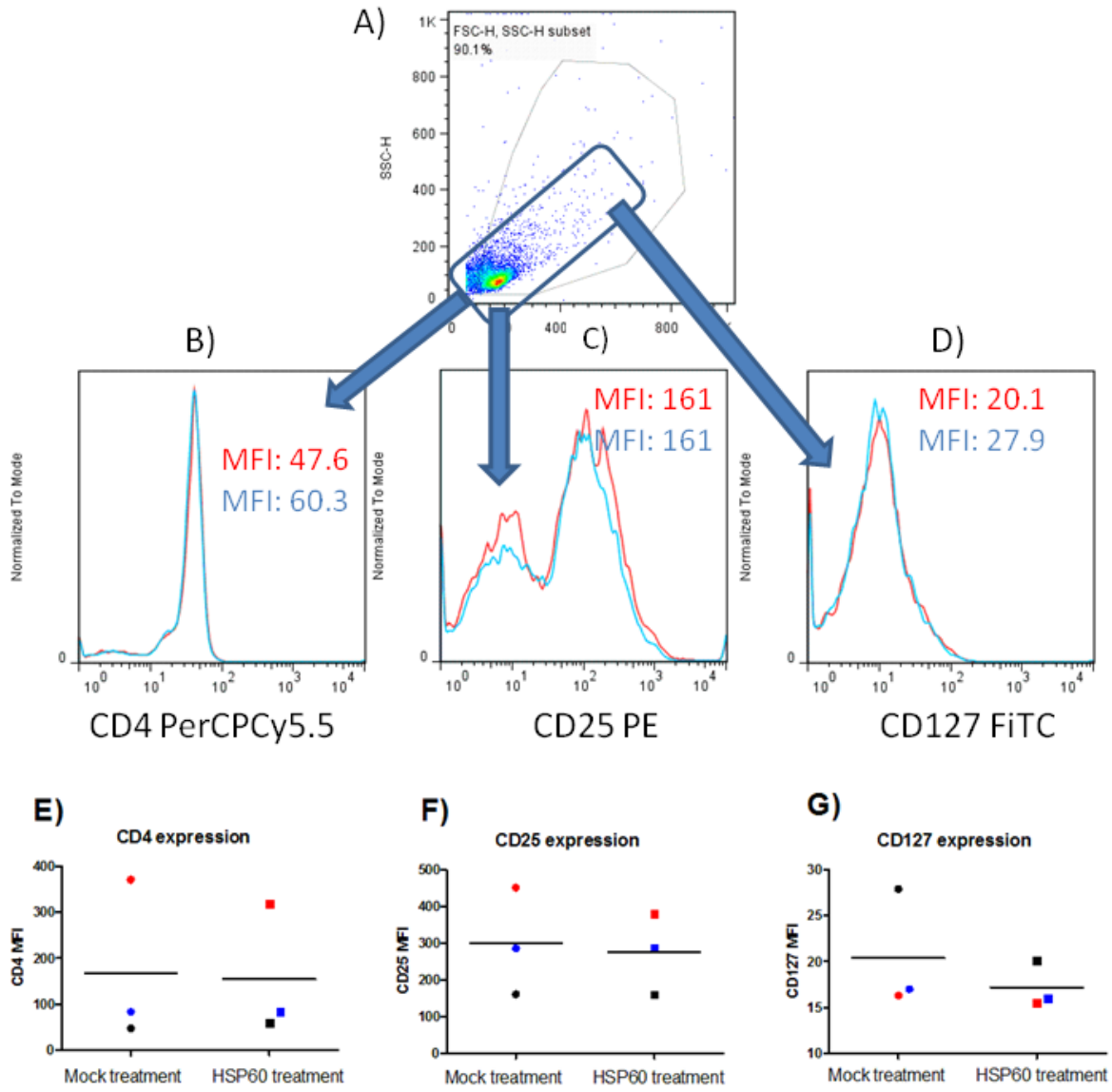


Figure 4.7 Two hours of HSP60 pre-treatment maintains the phenotype of regulatory T cells. This experiment was performed on cone blood and regulatory T cell isolation was performed as described in Figure 4.1. Regulatory T cells were pre-treated with 1 ng/ml of HSP60 for 2 hours before being washed away twice and cultured for 24 hours. Cells were harvested and surface staining of anti-CD4,CD25 and CD127 antibodies was performed. Data was acquired using FACS Calibur and analysed by FlowJo. Panel (A) shows the Forward versus Side scatter of regulatory T cells while Panel (B-D) show histogram of CD4 (B), CD25 (C) and CD127 (E). The red line represents mock treated regulatory T cells while the blue line represents HSP60 treated regulatory T cells. Panel (E-G) show average MFI of CD4 (E), CD25 (F) and CD127 (G) and each dot represents one donor with matching colour indicating the same donor.

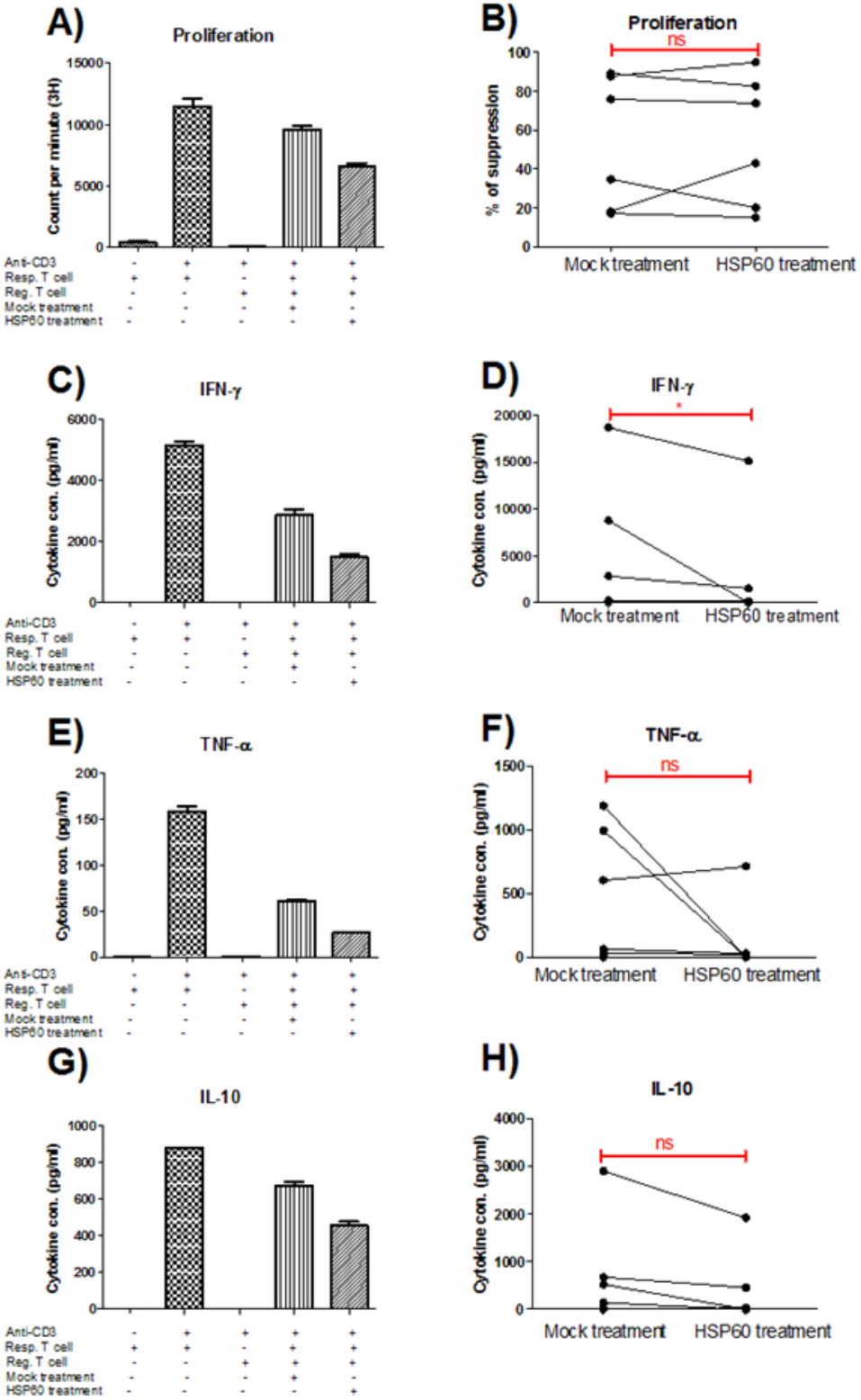

A)
Proliferation
Count per minute (3H)
15000
10000
5000
0
Anti-CD3
Resp. T cell
Reg. T cell
Mock treatment
HSP60 treatment
B)
Proliferation
ns
% of suppression
100
80
60
40
20
0
Mock treatment
HSP60 treatment
C)
IFN-γ
Cytokine con. (pg/ml)
6000
4000
2000
0
D)
IFN-γ
*
Cytokine con. (pg/ml)
20000
15000
10000
5000
0
Mock treatment
HSP60 treatment
E)
TNF-α
Cytokine con. (pg/ml)
200
150
100
50
0
F)
TNF-α
ns
Cytokine con. (pg/ml)
1500
1000
500
0
Mock treatment
HSP60 treatment
G)
IL-10
Cytokine con. (pg/ml)
1000
800
600
400
200
0
H)
IL-10
ns
Cytokine con. (pg/ml)
4000
3000
2000
1000
0
Mock treatment
HSP60 treatment

Figure 4.8. Two hours of HSP60 pre-treatment can enhance some aspects of regulatory T cell function. This experiment was performed on cone bloods and regulatory & responder T cell isolation was performed as shown in Figure 4.1. Regulatory T cells were treated with 1 ng/ml of HSP60 for 2 hours before being washed away twice and co-cultured with responder T cells to a ratio of 1 to 1 in the presence of 5 µg/ml plate-bound anti-CD3 antibodies. Cells were harvested after 96 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours while culture supernatants were harvested after 96 hours and cytokines were measured by ELISA. Panel (A) shows the proliferation of one donor and each bar represents a mean of 5 replicates and standard deviation as the error bar. On the other hand, Panel (B) demonstrates a mean percentage of suppression in multiple donors as each dot represents one donor. Panel ()show levels of cytokine measured from culture supernatant and each bar in Panel (C,E,G) represents an average of triplicate measurement and each dot in Panel (D,F,H) illustrates an average of one donor. The Wilcoxon signed rank test was used to compare between mock treatment and HSP60 treatment and * indicates p value <0.05 while ns indicates p value >0.05.

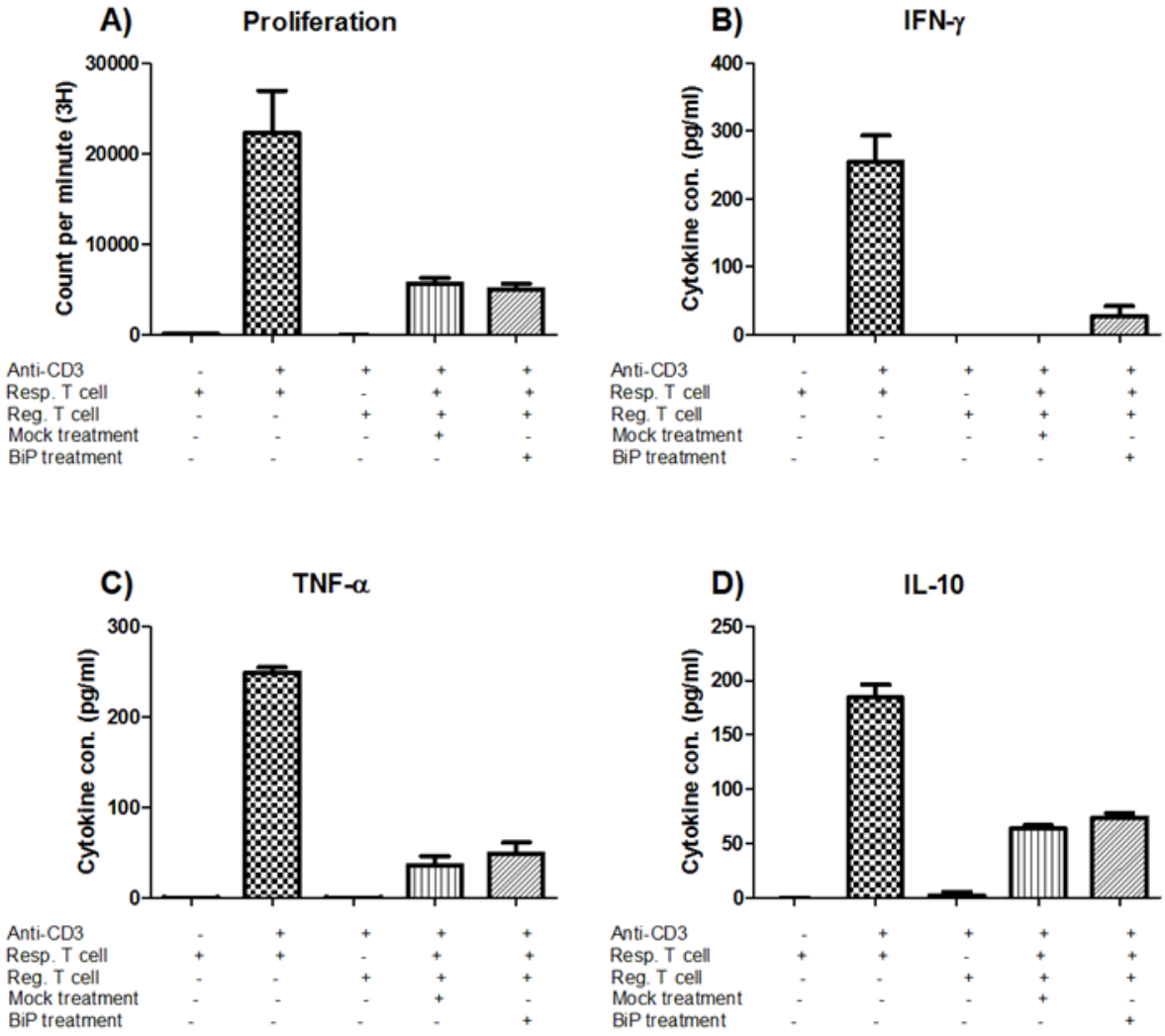


Figure 4.9 Two hours of BiP pre-treatment does not enhance regulatory T cell function. This experiment was performed on cone blood and regulatory & responder T cell isolation was performed as shown in Figure 4.1. Regulatory T cells were treated with 1 ng/ml of HSP60 for 2 hours before being washed away twice and co-cultured with responder T cells to a ratio of 1 to 1 in the presence of 5 µg/ml plate-bound anti-CD3 antibodies. Cells were harvested after 96 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours while culture supernatants were harvested after 96 hours and cytokines were measured by ELISA. Each bar in Panel (A) represents a mean of 5 replicates for proliferation while each bar of cytokine measurement in (B-D) is a mean of 3 replicates and each error bar represents standard deviation.

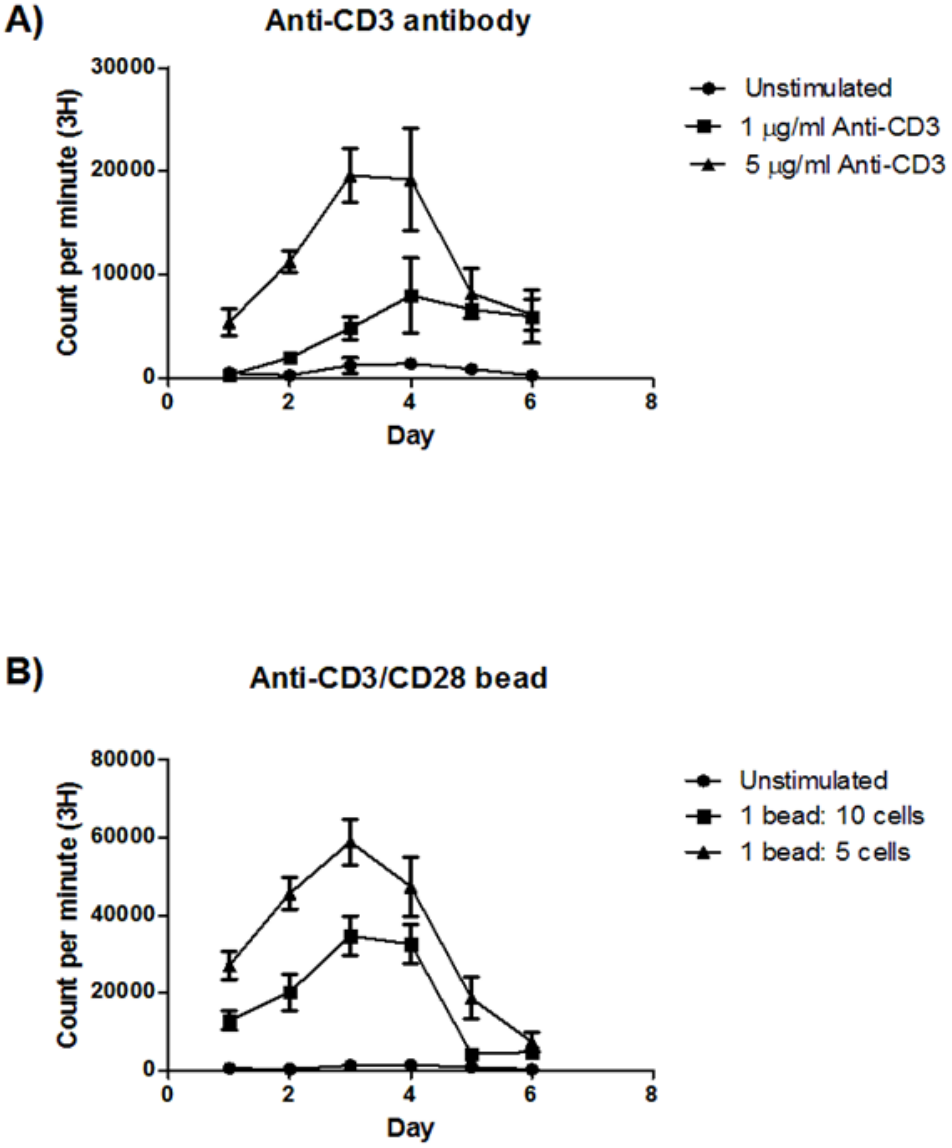


Figure 4.10 Peak responder T cell proliferation in the presence of various doses of anti-CD3 antibodies and anti-CD3/CD28 beads. This experiment was performed on cone bloods and responder T cell isolation was performed as described in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS in the presence of either anti-CD3 antibody or anti-CD3/CD28 beads. Responder T cells were harvested after 96 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours. Each dot represents a mean of 5 cone bloods and each error bar represents standard error of mean of 5 donors. Panel (A) demonstrates responder T cell proliferation in the presence of anti-CD3 antibody while (B) shows responder T cell proliferation in the presence of anti-CD3/CD28 beads.

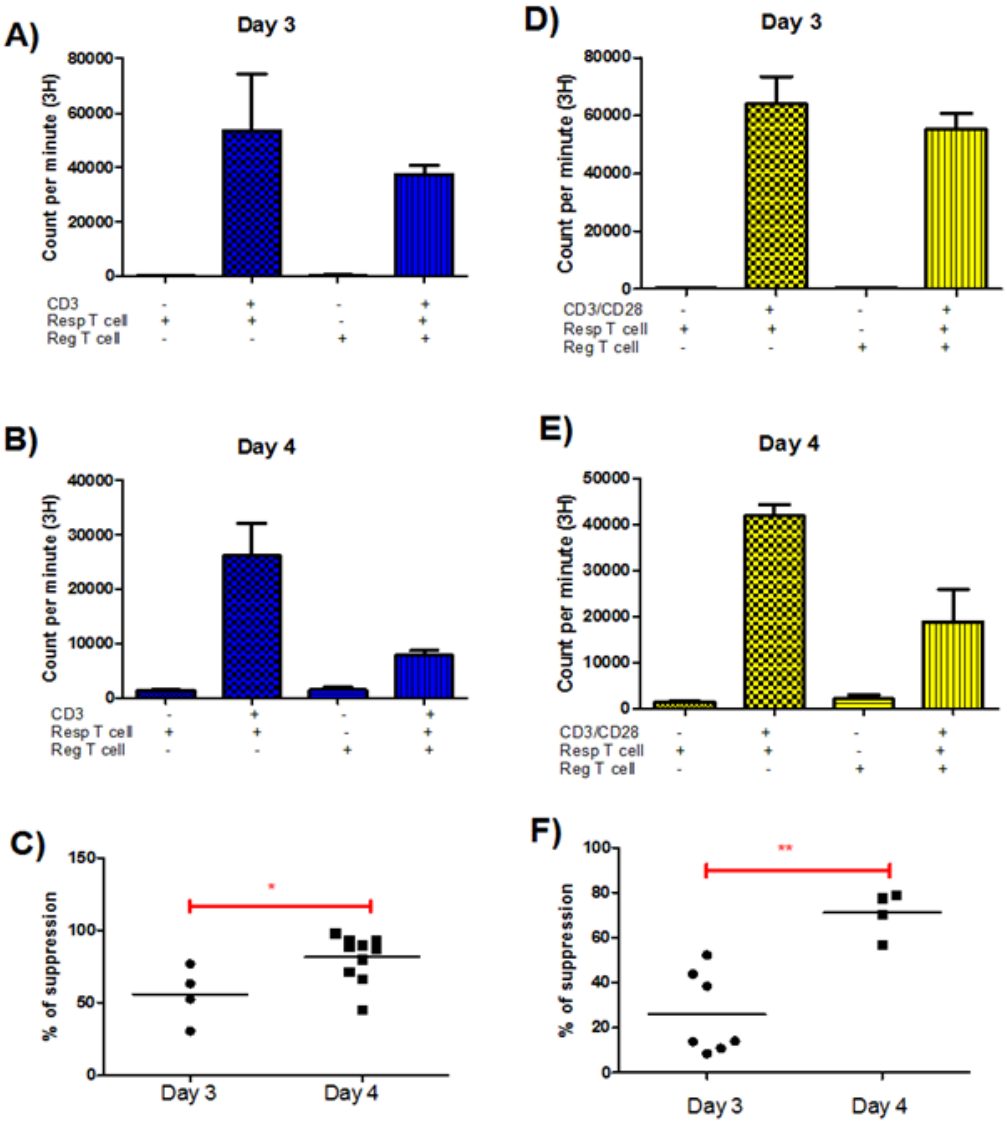


Figure 4.11 Optimum suppression was achieved on Day 4. This experiment was performed on cone bloods and responder and regulatory T cell isolation was performed as described in Figure 4.1. Responder and regulatory T cells were co-cultured with RPMI media supplemented with 10% FCS in the presence of either 5 µg/ml plate-bound anti-CD3 antibodies (A&B) or 1 anti-CD3/CD28 bead to 5 cells (D&E). Cell were harvested after 72 (A&D) or 96 (B&E) hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours. Each bar in (A-E) represents a mean of 5 replicates of one cone blood while each dot in (C&F) represents percentage of suppression of cone bloods from multiple donors. The Mann Whitney test was used to compare the percentage of suppression between Day 3 and Day 4 in the presence of both stimuli. * indicates p value <0.05 while ** indicates p value <0.01.

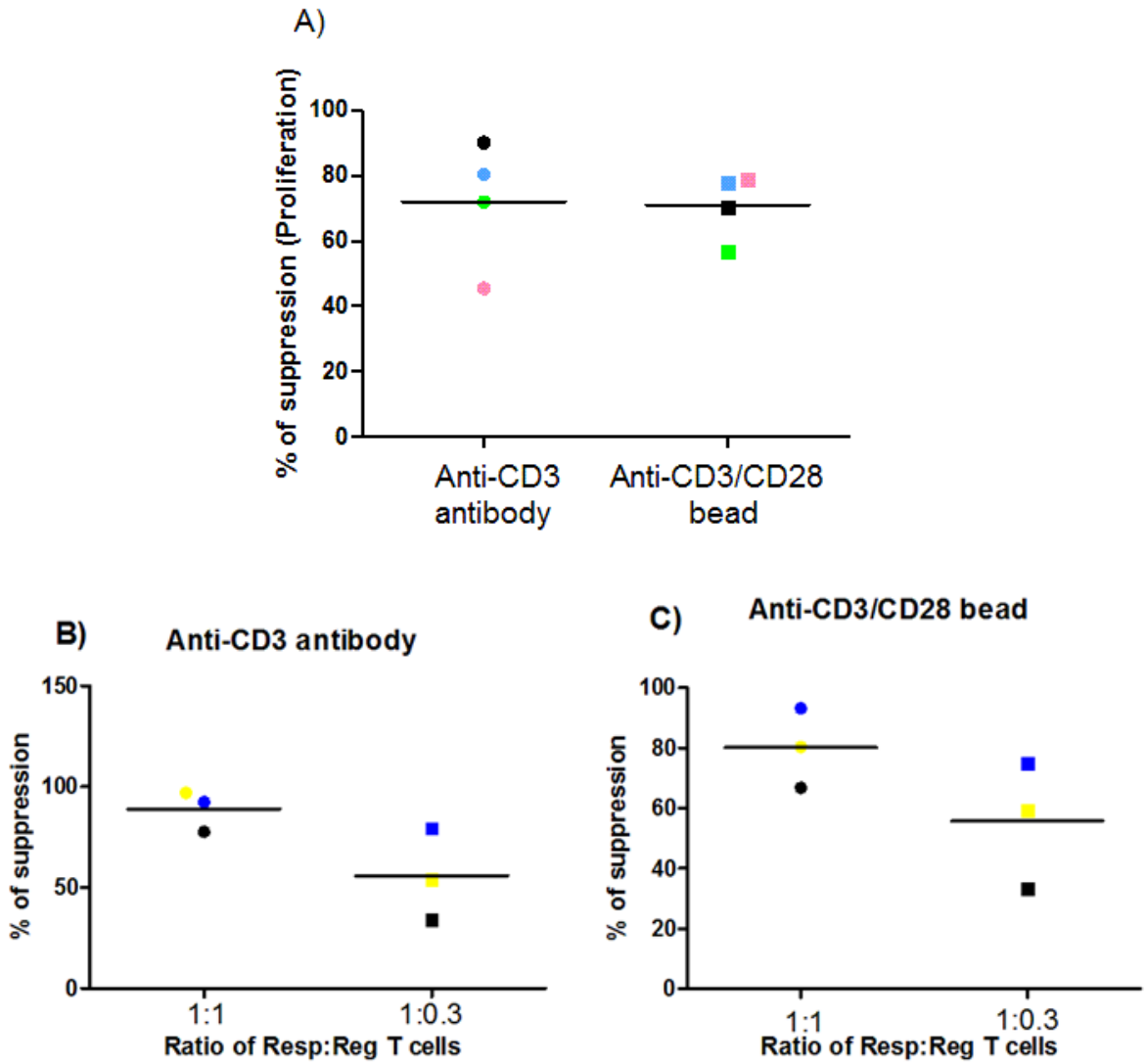
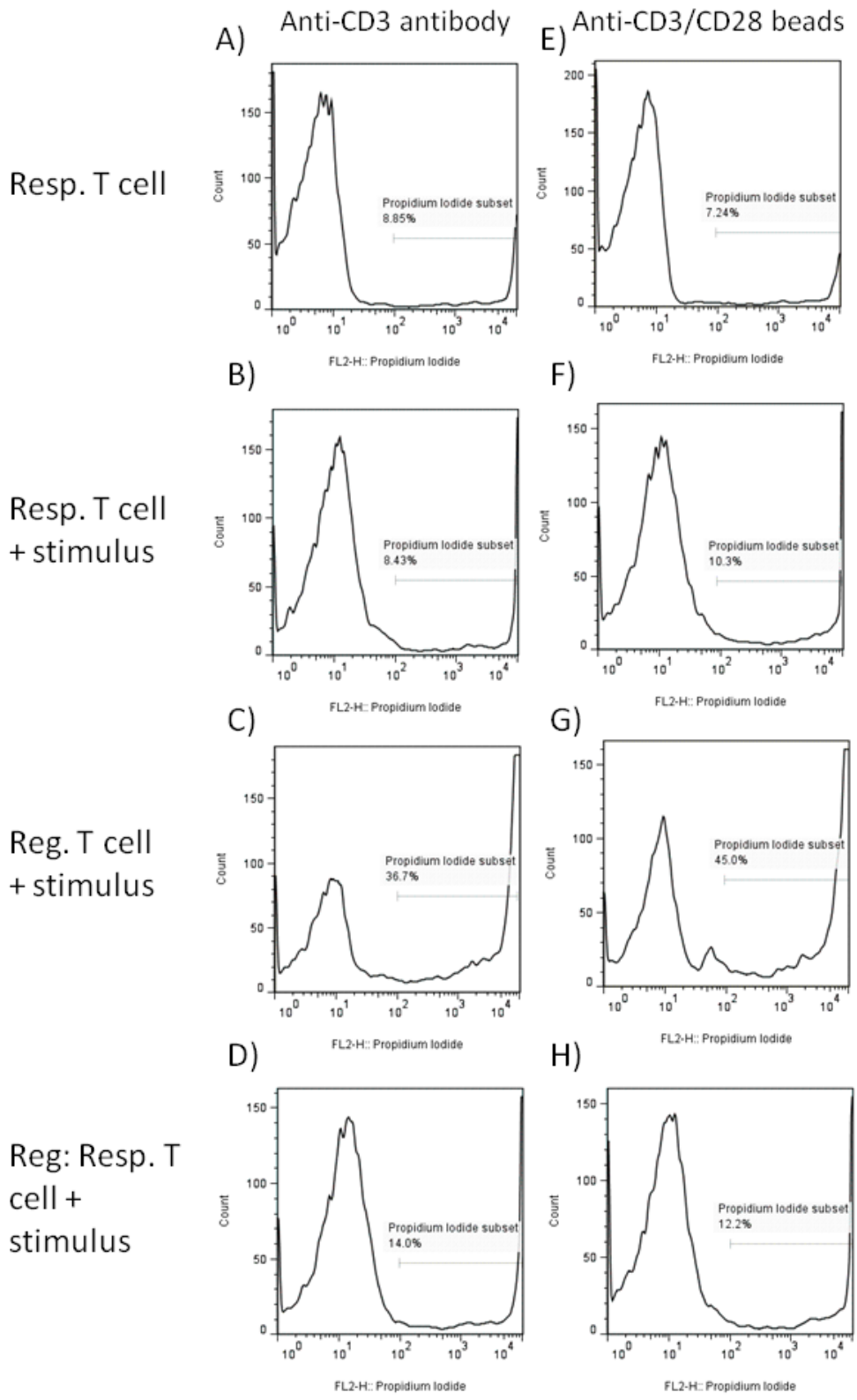


Figure 4.12. Sub-optimal suppression was achieved with ratio of 1:0.3 (responder to regulatory T cells) compared to 1:1 ratio. This experiment was performed on cone bloods and responder and regulatory T cell isolation was performed as shown in Figure 4.1. Responder and regulatory T cells were co-cultured with RPMI media supplemented with 10% FCS in the presence of either 5 µg/ml plate-bound anti-CD3 antibody or 1 anti-CD3/CD28 bead to 5 cells. Cell were harvested after 96 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours. Panel (A) shows the percentage of suppression in the ratio of 1:1 (responder to regulatory T cells) while Panel (B) and (C) show the percentage of suppression comparing between 1:1 ratio and 1:0.3 ratio. Each dot in (A-C) represents one donor and a matching colour indicates a matching donor.


Anti-CD3 antibody
Anti-CD3/CD28 beads
A)
E)
B)
F)
C)
G)
D)
H)
Resp. T cell
Resp. T cell + stimulus
Reg. T cell + stimulus
Reg: Resp. T cell + stimulus
Count
FL2-H:: Propidium Iodide
Propidium Iodide subset
8.85%
7.24%
8.43%
10.3%
36.7%
45.0%
14.0%
12.2%

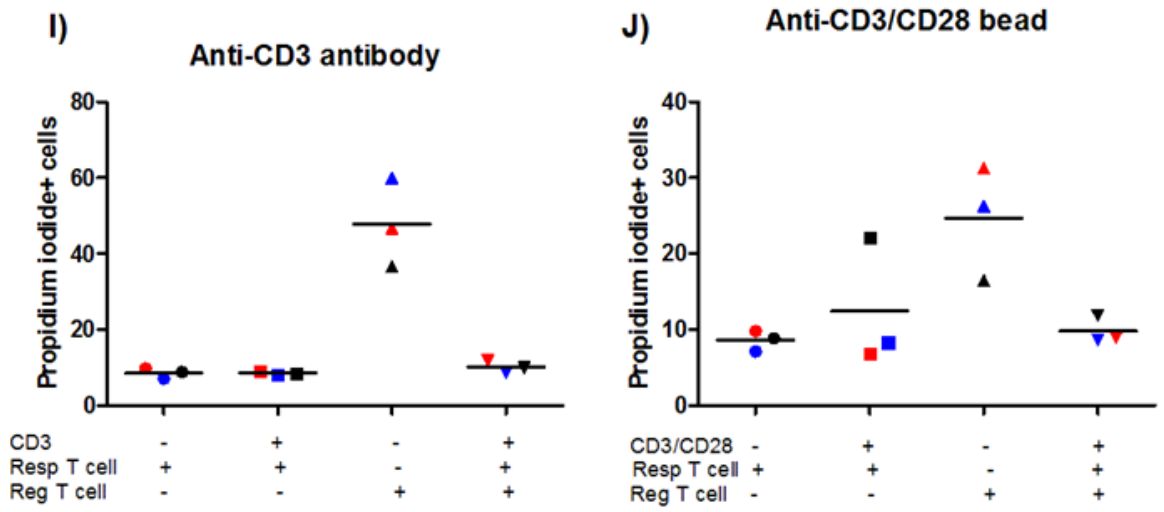


Figure 4.13 Decreased proliferation of responder T cells in the co-culture with regulatory T cells is not due to an increase in cell death. This experiment was performed on cone bloods and responder and regulatory T cell isolation was performed as shown in Figure 4.1. Cells were cultured with RPMI media supplemented with 10% FCS in the presence of either anti-CD3 antibodies or anti-CD3/CD28 beads. Cell suspension was harvested after 96 hours and stained with propidium iodide, data was acquired using FACS Calibur and analysed using FlowJo. Panel (A-D) show histogram of propidium iodide in the presence of anti-CD3 antibody in one donor and multiple donor in (I) while Panel (E-H) show histograms of propidium iodide in the presence of anti-CD3/CD28 beads in one donor and multiple donor in (J). Matching colours in (I&J) indicate the same donors.

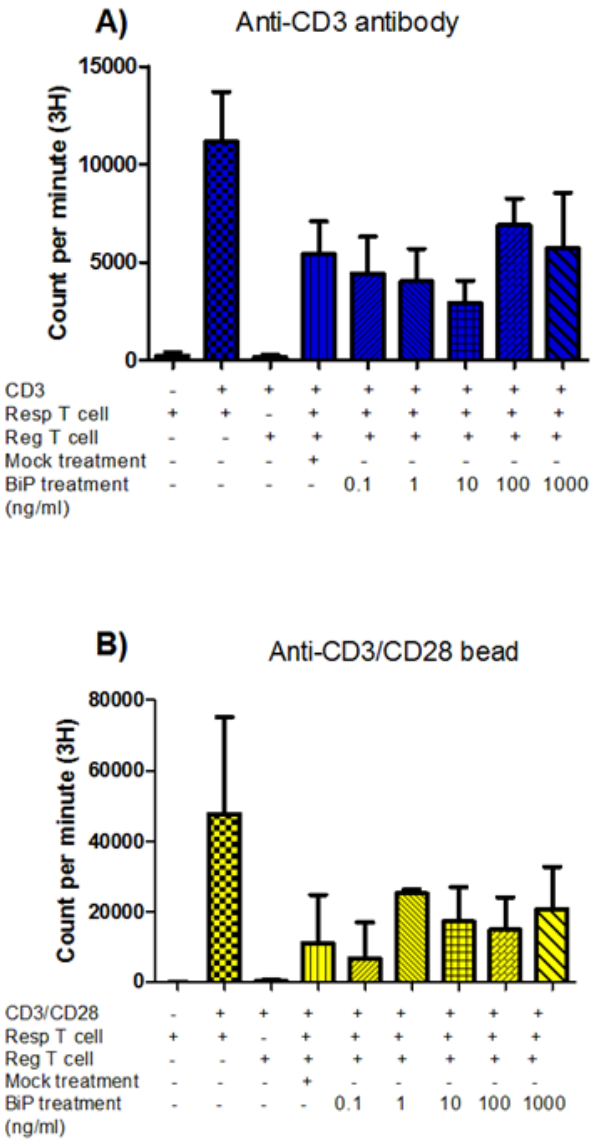


Figure 4.14. 10 ng/ml is the most potent dose of BiP as demonstrated by the greatest reduction in proliferation. This experiment was performed on cone blood and regulatory & responder T cell isolation was performed as shown in Figure 4.1. Regulatory T cells were treated with various doses of BiP for 2 hours before being washed away twice and co-cultured with responder T cells to a ratio of 0.3 to 1 in the presence of 5 µg/ml plate-bound anti-CD3 antibody (A) or 1 anti-CD3/CD28 bead to 5 cells (B). Cells were harvested after 96 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours. Each bar in (A&B) represents a mean of 5 replicates for proliferation and this figure is representative of 2 cone bloods.

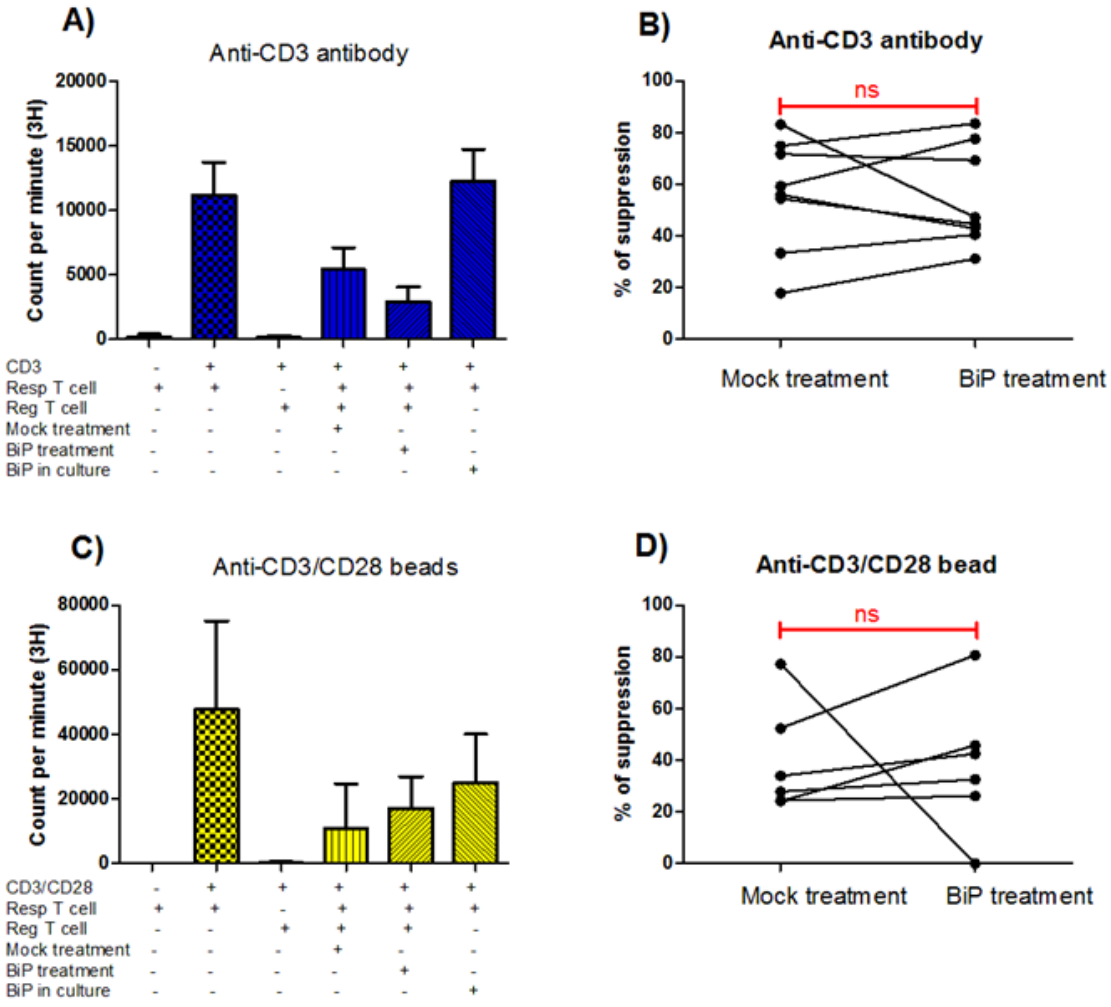


Figure 4.15. Two hours of BiP pre-treatment does not enhance regulatory T cell function as measured by proliferation of responder T cells in the co-culture. This experiment was performed on cone blood and regulatory & responder T cell isolation was performed as shown in Figure 4.1. Regulatory T cells were treated with 10 ng/ml of BiP for 2 hours before being washed away twice and co-cultured with responder T cells to a ratio of 0.3 to 1 in the presence of 5 µg/ml plate-bound anti-CD3 antibodies (A) or 1 anti-CD3/CD28 bead to 5 cells (B). Cells were harvested after 96 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours. Each bar in (A&B) represents a mean of 5 replicates for proliferation and proliferation data was converted into the percentage of suppression. Each dot represents the percentage of suppression for one cone blood of 8 donors (C) or 6 donors (D). The Wilcoxon signed rank test was used to compare percentage of suppression between mock treatment and BiP treatment of regulatory T cells and no significant difference was observed.

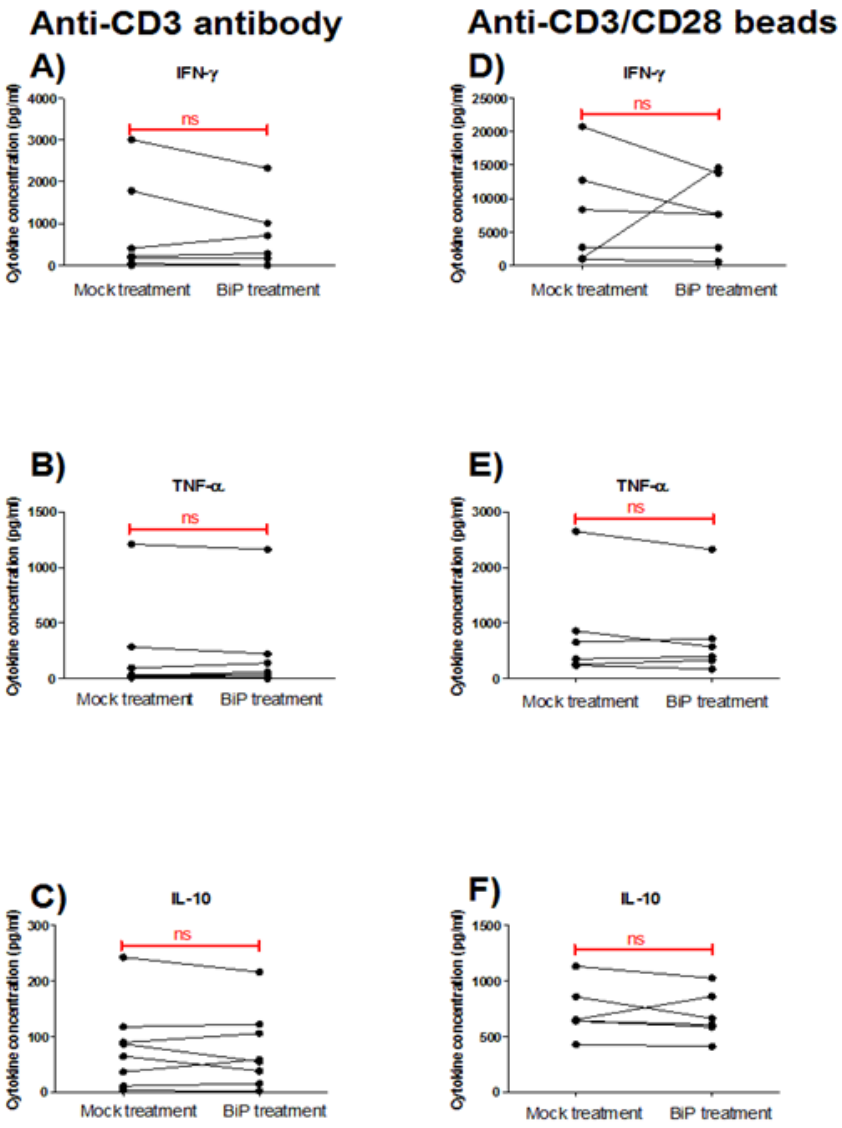


Figure 4.16. Two hours of BiP pre-treatment does not enhance regulatory T cell function as measured by cytokine profile from the co-culture. This experiment was performed on cone bloods and regulatory & responder T cell isolation was performed as described in Figure 4.1. Regulatory T cells were treated with 10 ng/ml of BiP for 2 hours before being washed away twice and co-cultured with responder T cells with the ratio of 0.3 to 1 in the presence of 5 µg/ml plate-bound anti-CD3 antibody (A-C) or 1 anti-CD3/CD28 bead to 5 cells (D-F). Culture supernatants were harvested after 96 hours and cytokines were measured by ELISA and each dot represents a mean of triplicate cytokine measurement of one cone blood. The Wilcoxon signed rank test was used to compare cytokine levels from the co-culture between mock treatment and BiP treatment of regulatory T cells and ns indicates p value >0.05.

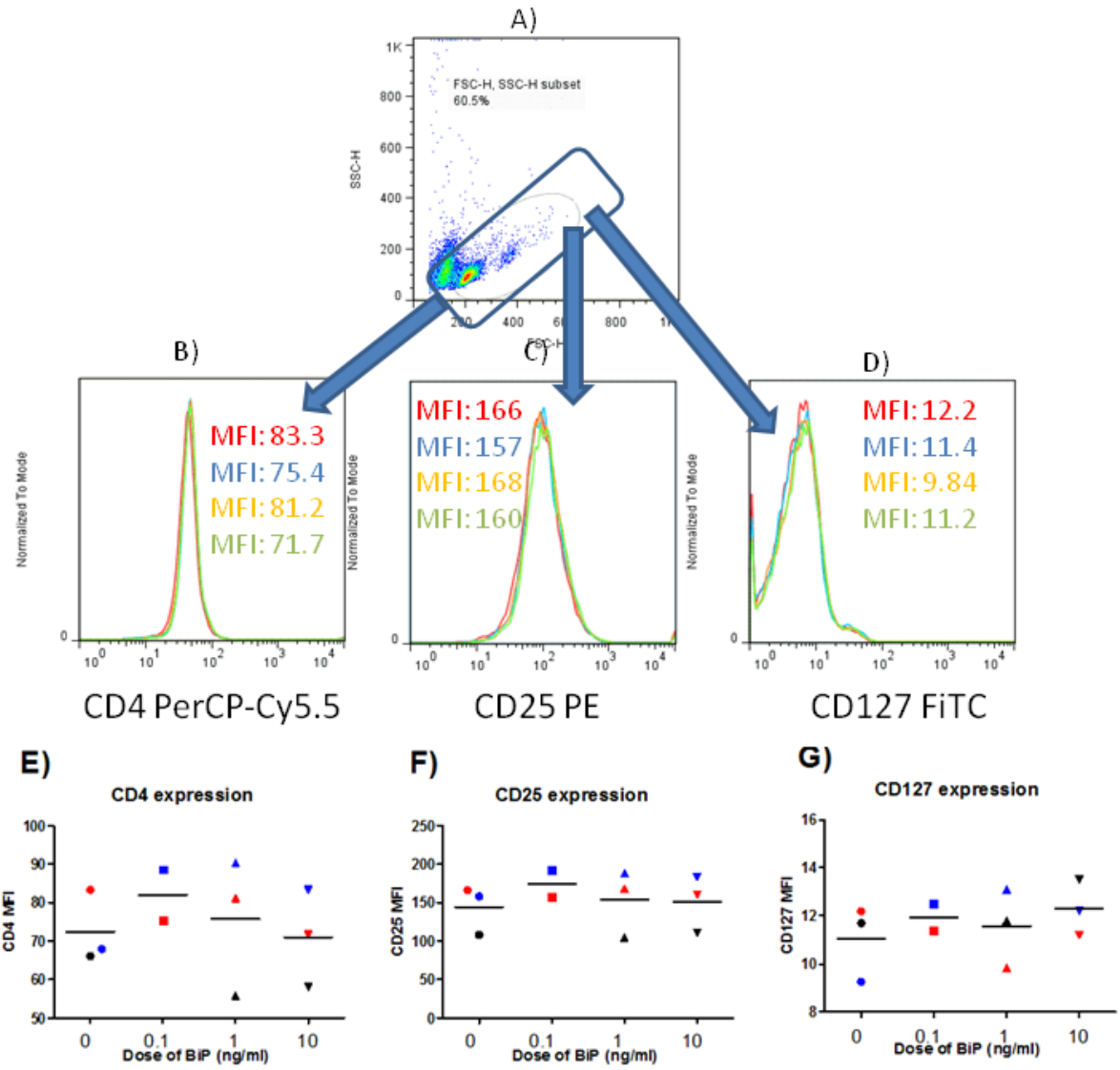


Figure 4.17. Two hours of BiP pre-treatment maintains regulatory T cell phenotype. This experiment was performed on cone blood and regulator T cell isolation was performed as shown in Figure 4.1. Regulatory T cells were pre-treated with 0.1,1 and 10 ng/ml of BiP for 2 hours before being washed away twice and cultured for 24 hours. Cells were harvested and surface staining of anti-CD4,CD25 and CD127 antibodies was performed. Data was acquired using FACS Calibur and analysed by FlowJo. Panel (A) shows Forward versus Side scatter of regulatory T cells while Panel (B-D) show histogram of CD4 (B), CD25 (C) and CD127 (E). The red line represents mock treated regulatory T cells while the rest represents BiP treated regulatory T cells: 0.1 ng/ml of BiP treated regulatory T cell (blue), 1 ng/ml of BiP treated regulatory T cell (orange) and 10 ng/ml of BiP (green). Panel (E-G) show the average MFI of CD4 (E), CD25 (F) and CD127 (G) and each dot represents one donor with a matching colour indicates the same donor.

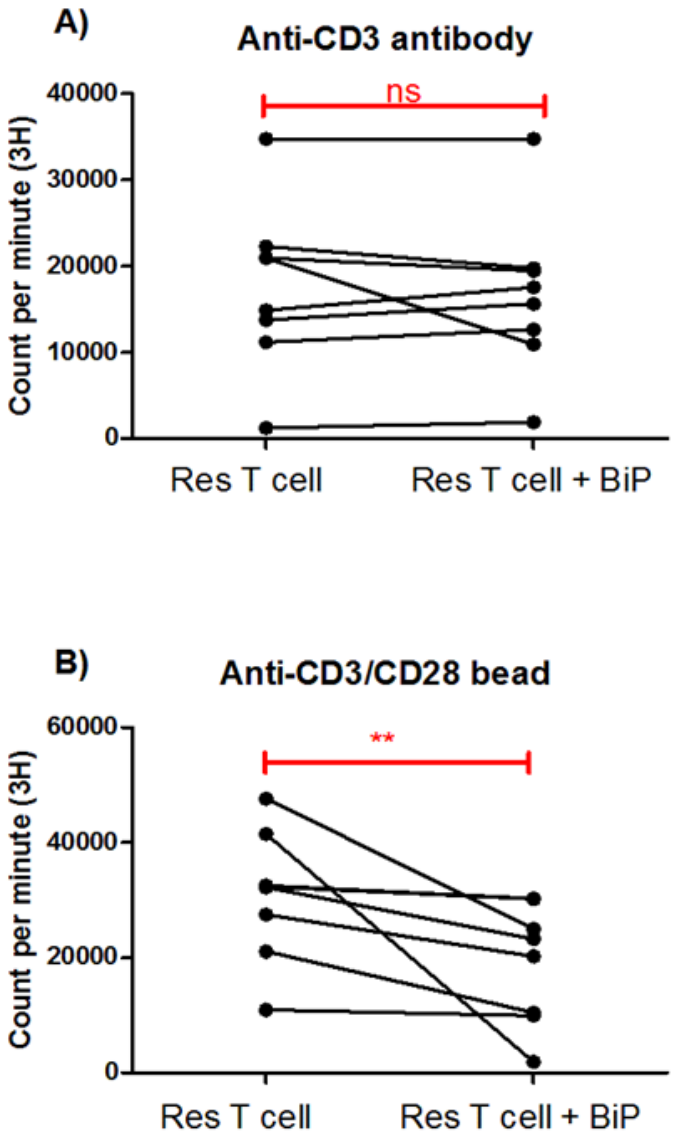


Figure 4.18 BiP can reduce responder T cell proliferation in the presence of anti-CD3/CD28 beads. This experiment was performed on cone blood and responder T cell isolation was performed as described in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 96 hours in the presence of 5 µg/ml plate-bound anti-CD3 antibodies (A) or 1 anti-CD3/CD28 beads to 5 cells (B) with or without 10 ng/ml of BiP. Responder T cells were harvested after 96 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours. Each dot represents a mean of 5 replicates from 8 donors. Responder T cell proliferation was compared with cultured in the absence or presence of BiP using the Wilcoxon signed rank test. ** indicates p value <0.01 while ns indicates p value >0.05.

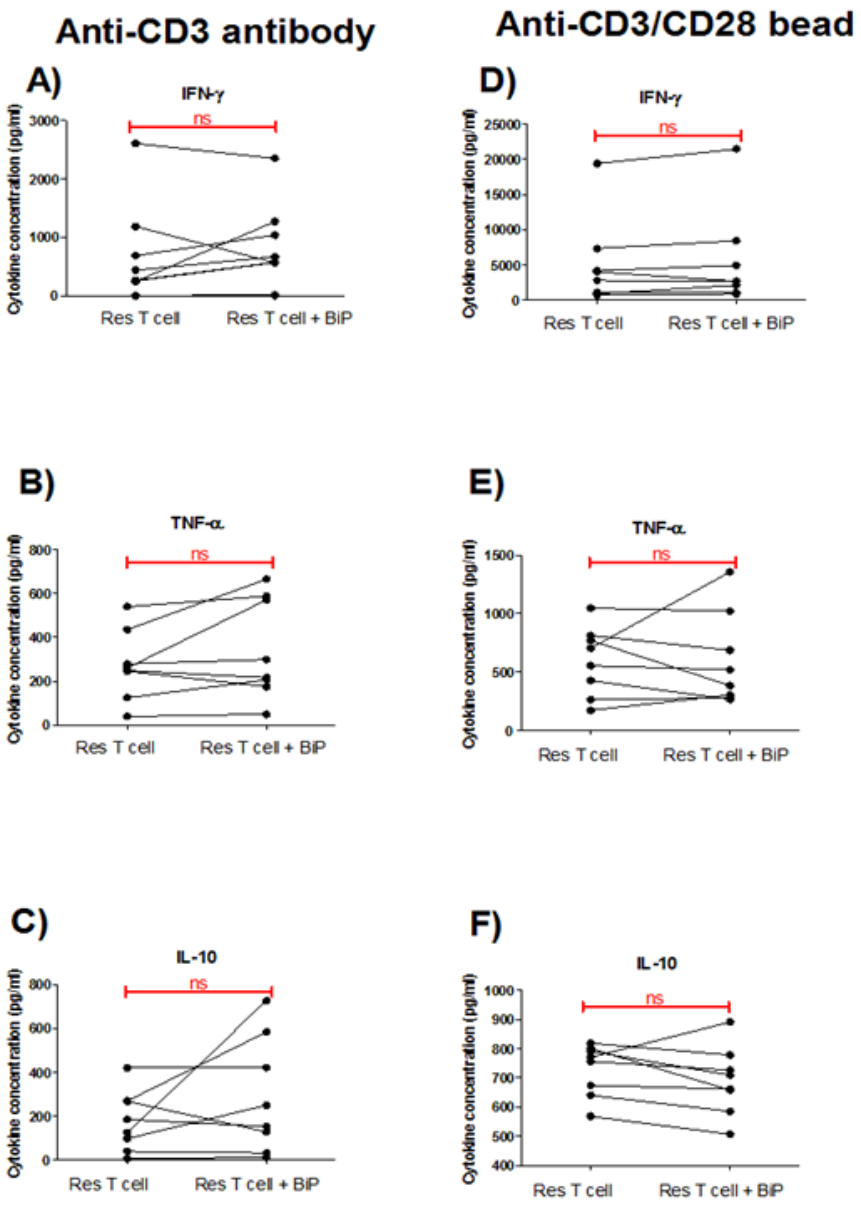


Figure 4.19 BiP does not induce any changes in supernatant cytokines secreted from responder T cells. This experiment was performed on cone bloods and responder T cell isolation was performed as described in Figure 4.1. Cells were cultured with RPMI media supplemented with 10% FCS for 96 hours in the presence of 5 µg/ml plate-bound anti-CD3 antibodies (A-D) or 1 anti-CD3/CD28 bead to 5 cells (E-H) with or without 10 ng/ml BiP. Culture supernatants were harvested after 96 hours and cytokines were measured by ELISA. Each dot represents a mean of 3 replicates of one or multiple donor. Cytokine levels of IFN-γ, TNF-α and IL-10 were compared between responder T cells cultured in the absence or presence of BiP using the Wilcoxon signed rank test and ns indicates p value >0.05.

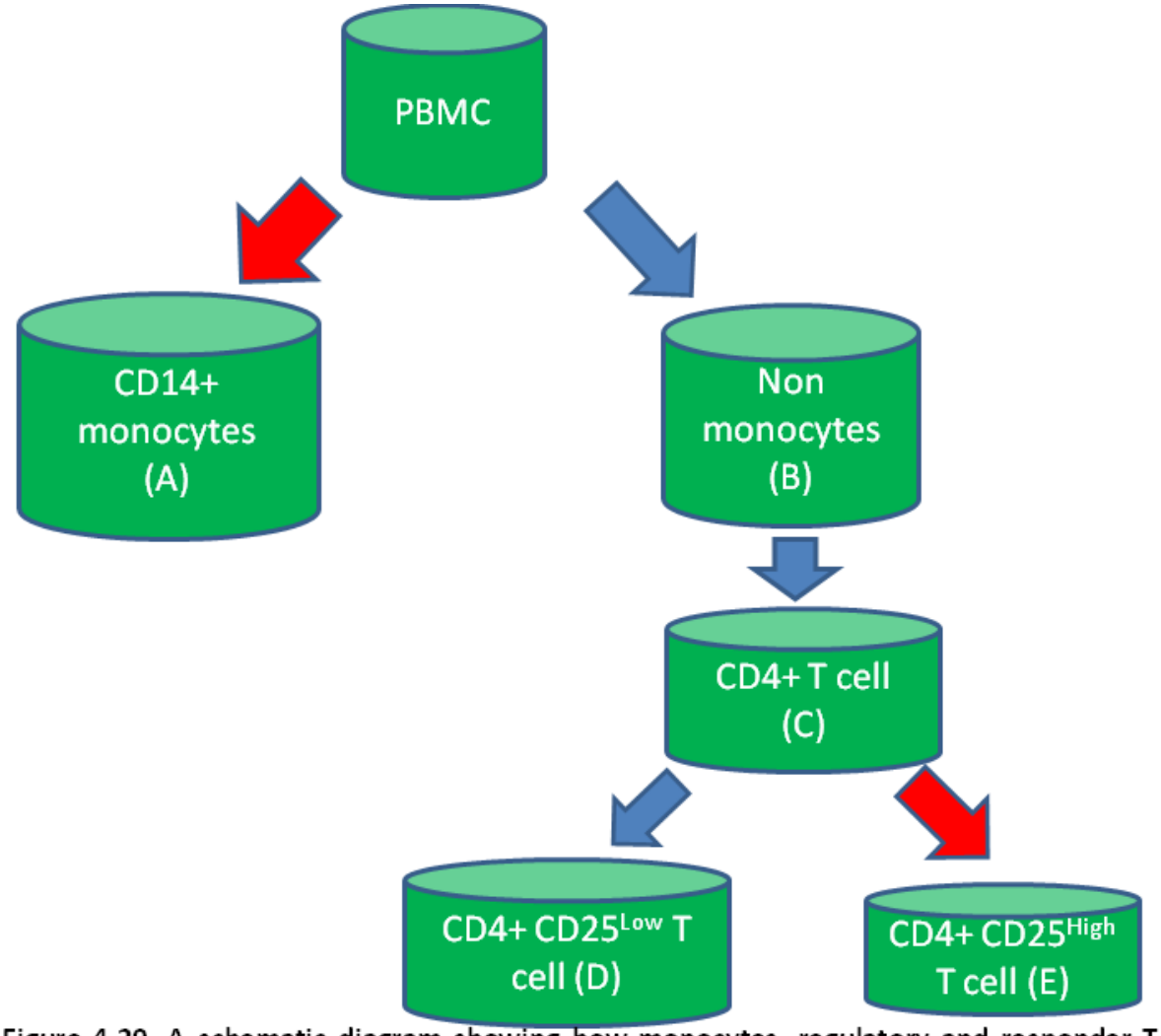
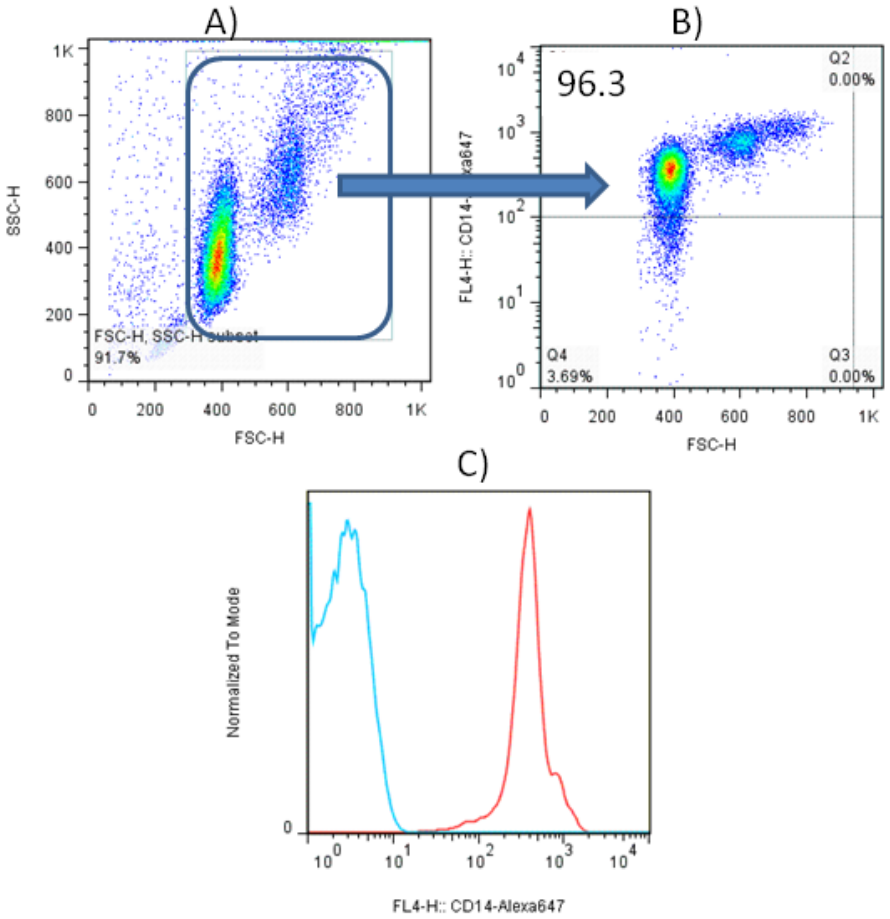


Figure 4.20. A schematic diagram showing how monocytes, regulatory and responder T cells were isolated using Human C14 Microbeads and CD4+CD25+ Regulatory T cell Isolation Kit. PBMCs were obtained using density gradient centrifugation using Lymphoprep. After that, PBMCs were incubated with CD14+ Microbeads. Then, CD14+ monocytes were isolated using LS column. CD14+ monocytes (A) were collected by washing away cells bound to the LD column while non monocytes (B) were collected as flow through of the LS column. After that, the non monocyte fraction was incubated with CD4+ T cell Biotin-Antibody Cocktail and Anti-Biotin Microbeads. Then, CD4+ T cells were isolated using the LD column. CD4+ T cells (C) were collected as flow through of the LD column. After that, CD4+ T cells were incubated with CD25 antibodies and isolated using the MS column (Miltenyi Biotech). Responder T cells were collected as flow through of the MS column (D) while regulatory T cells were collected by washing away cells bound to the first MS column (E). Purity staining was performed for monocytes (anti-CD14 antibody) and responder and regulatory T cells (anti-CD4, anti-CD25 and anti-CD127 antibodies). The red arrows indicate positive selection while blue arrows indicate negative selection.



Monocytes	CD14+ (%±SD)
N=5	73.6±21

Figure 4.21. High purity of monocytes after isolation using CD14 Mircobeads. This experiment was performed on cone bloods, and monocytes and responder T cell isolation was performed as shown in Figure 4.19. After that, cells were stained with human anti-CD14 antibodies. Cells were acquired using FACS Calibur and data was analysed using FlowJo. Panel (A) shows Forward v Side Scatter of monocytes after isolation and (B) shows CD14 expression. Panel (C) shows a histogram of CD14 comparing antibody staining (red line) and isotype control (blue line). The table demonstrates the mean frequency of CD14+ cells±standard deviation.

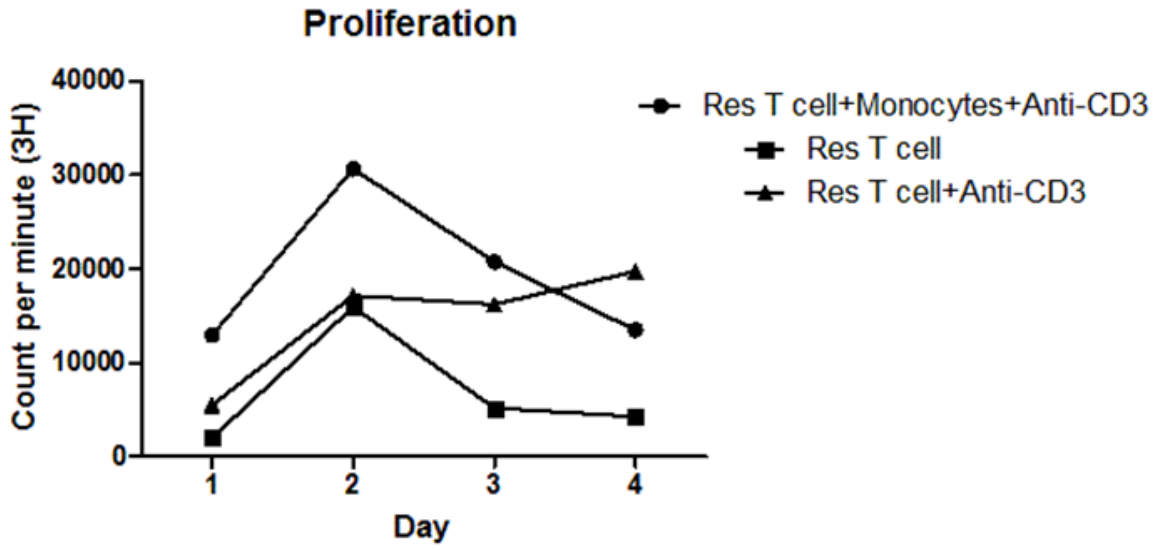
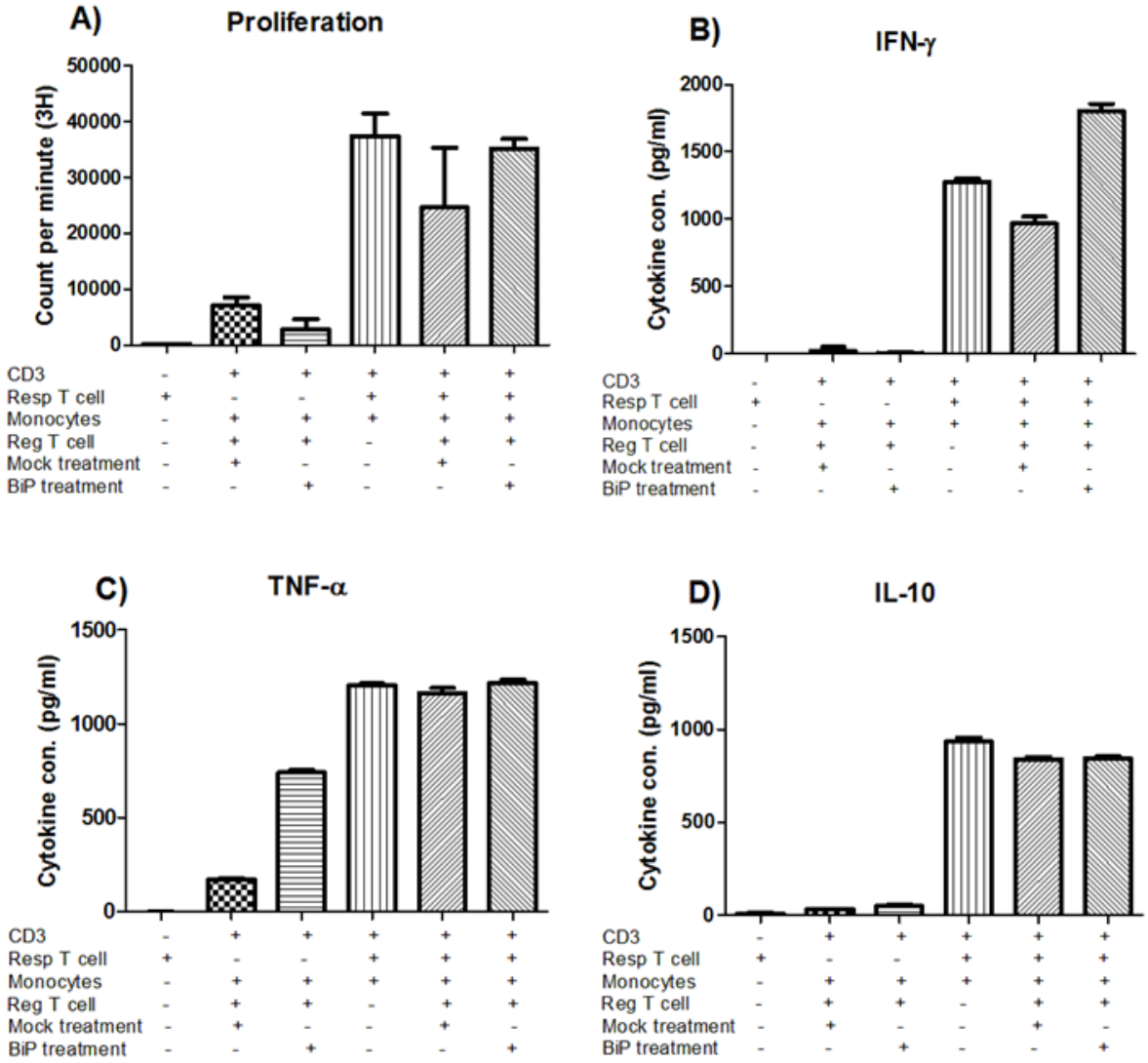
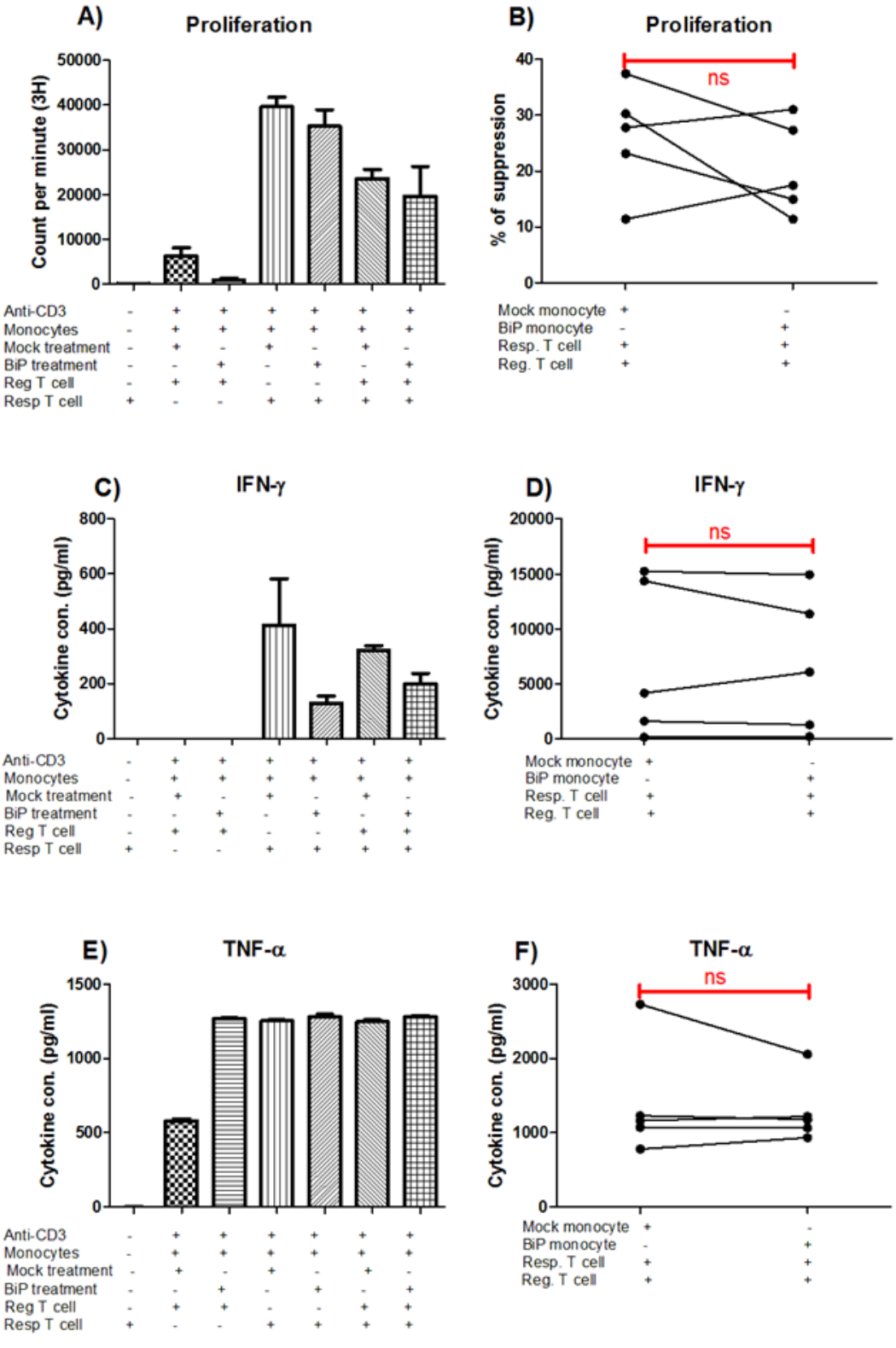


Figure 4.22. Responder T cell proliferation peaks at Day 2 in the presence of monocytes and anti-CD3 antibodies. This experiment was performed on cone bloods and monocytes and responder T cell isolation was performed as shown in Figure 4.19. Responder T cells and monocytes were cultured in the ratio of 1:0.3 with RPMI media supplemented with 10% FCS in the presence of 5 µg/ml plate-bound anti-CD3 antibodies. Cells were harvested every day for 4 days and proliferation was measured by tritiated thymidine incorporation for 16-18 hours. Each dot represents a mean of 2 cone bloods.



Reg. T cell treatment (n=3)	Proliferation (% of suppression)	IFN-γ	TNF-α	IL-10
Mock treatment	28.4±12	4662±3021	1208.5±48	532.9±153
BiP treatment	18.4±6	5154.3±2740	1189.3±42	517.6±163

Figure 4.23 Two hours of BiP pre-treatment does not enhance regulatory T cell function even in the presence of monocytes. This experiment was performed on cone bloods and monocytes. Responder and regulatory T cell isolations were performed as described in Figure 4.19. Regulatory T cells were treated with 10 ng/ml of BiP for 2 hours before being washed away twice and co-cultured with monocytes in the ratio of 1:1 for 24 hours in the presence of 5 µg/ml plate-bound anti-CD3 antibodies. After 24 hours, responder T cells were added into the co-culture and the final ratio of cells was (responder:regulatory:monocytes) 1:0.3:0.3. Cells and culture supernatants were harvested after 48 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours while cytokines were measured by ELISA. Each bar in (A) represents a mean of 5 replicates while each bar in (B-D) represents a mean of triplicates. The table demonstrates the mean percentage of suppression and supernatant cytokine levels±standard deviation.


A)
Proliferation
Count per minute (3H)
50000
40000
30000
20000
10000
0
Anti-CD3 - + + + + + +
Monocytes - + + + + + +
Mock treatment - + - + - + -
BiP treatment - - + - + - +
Reg T cell - + + - - + +
Resp T cell + - - + + + +
B)
Proliferation
% of suppression
40
30
20
10
0
ns
Mock monocyte + -
BiP monocyte - +
Resp. T cell + +
Reg. T cell + +
C)
IFN-γ
Cytokine con. (pg/ml)
800
600
400
200
0
Anti-CD3 - + + + + + +
Monocytes - + + + + + +
Mock treatment - + - + - + -
BiP treatment - - + - + - +
Reg T cell - + + - - + +
Resp T cell + - - + + + +
D)
IFN-γ
Cytokine con. (pg/ml)
20000
15000
10000
5000
0
ns
Mock monocyte + -
BiP monocyte - +
Resp. T cell + +
Reg. T cell + +
E)
TNF-α
Cytokine con. (pg/ml)
1500
1000
500
0
Anti-CD3 - + + + + + +
Monocytes - + + + + + +
Mock treatment - + - + - + -
BiP treatment - - + - + - +
Reg T cell - + + - - + +
Resp T cell + - - + + + +
F)
TNF-α
Cytokine con. (pg/ml)
3000
2000
1000
0
ns
Mock monocyte + -
BiP monocyte - +
Resp. T cell + +
Reg. T cell + +

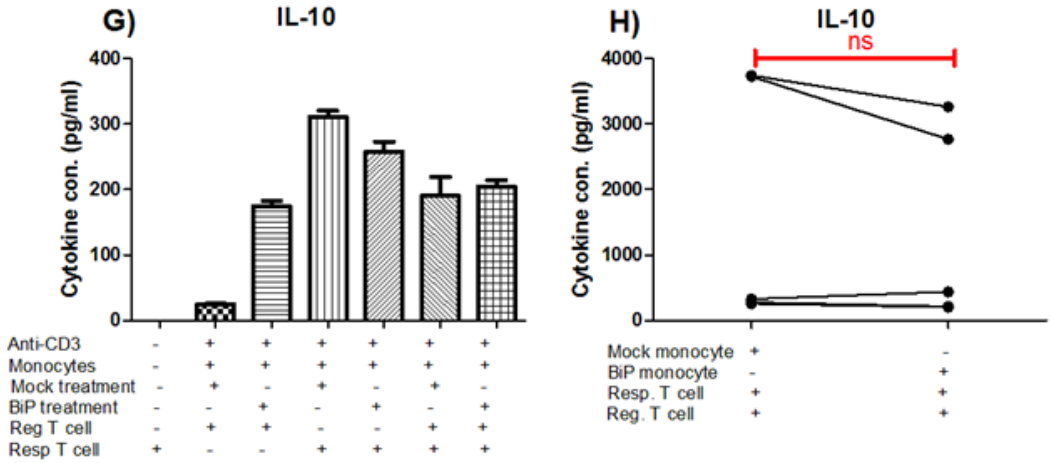


Figure 4.24 Two hours of BiP pre-treatment of monocytes does not enhance regulatory T cell function. This experiment was performed on cone bloods and monocytes. Responder and regulatory T cell isolations were performed as described in Figure 4.19. Monocytes were treated with 10 ng/ml of BiP for 2 hours before being washed away twice and co-cultured with regulatory T cells in the ratio of 1:1 for 24 hours in the presence of 5 µg/ml plate-bound anti-CD3 antibodies. After 24 hours, responder T cells were added into the co-culture and the final ratio of cells was (responder:regulatory:monocytes) 1:0.3:0.3. Cells and culture supernatants were harvested after 48 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours while cytokines were measured by ELISA. Panel(A) shows the proliferation for one donor and each bar represents a mean of 5 replicates and standard deviation as the error bar. On the other hand, Panel (B) demonstrates a mean percentage of suppression in multiple donors as each dot represents one donor. Panel (C-H) show levels of cytokine measured from culture supernatant and each bar in Panel (C-G) represents a mean of triplicate measurements and each dot in Panel (D-H) illustrates a mean of one out of 5 donor. The Wilcoxon signed rank test was used to compare between mock treatment and BiP treatment and ns indicates p value >0.05.

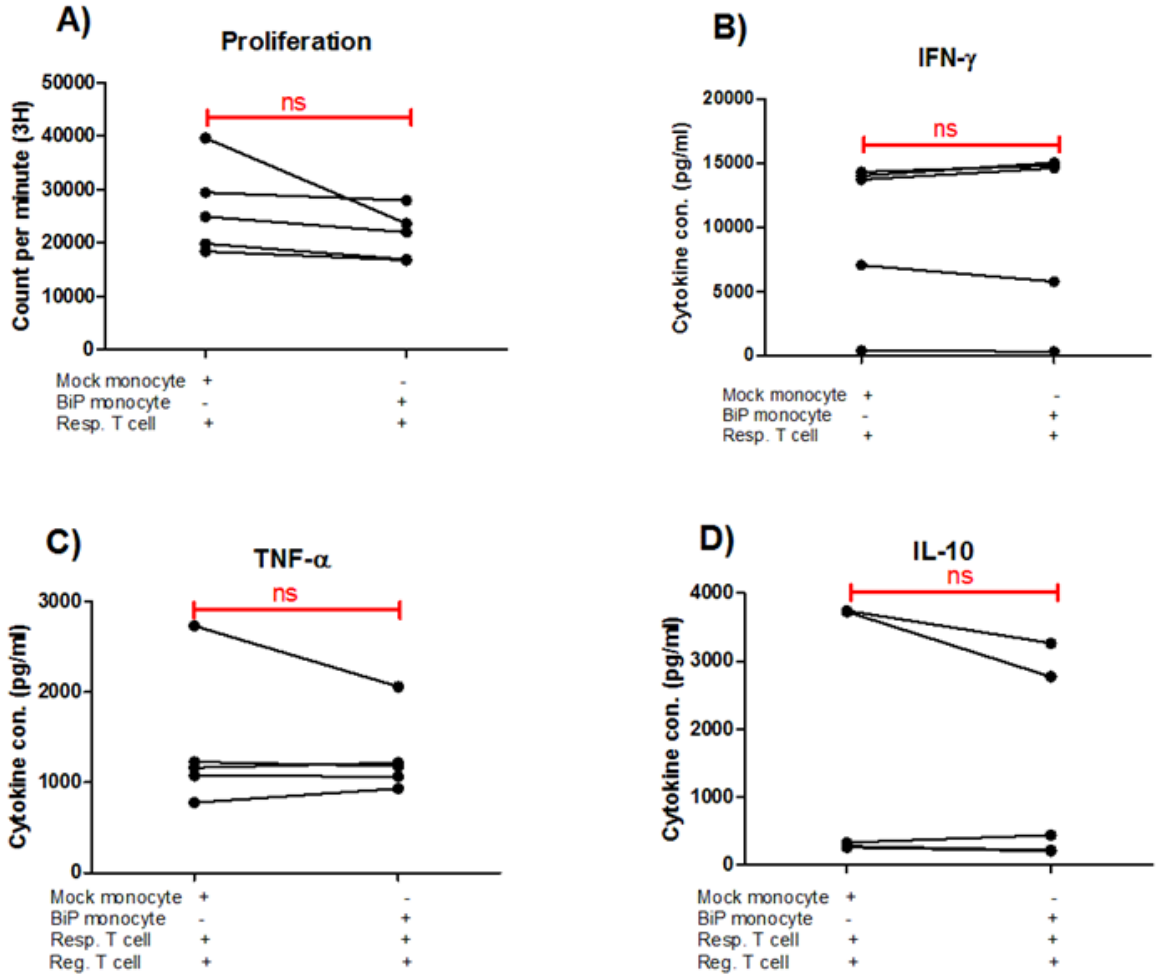


Figure 4.25 Two hours of BiP pre-treatment of monocytes reduce proliferation of responder T cells although the difference is statistically insignificant (p=0.06). This experiment was performed on cone bloods and monocytes, and responder T cell isolations were performed as shown in Figure 4.19. Monocytes were treated with 10 ng/ml of BiP for 2 hours before being washed away twice and co-cultured with responder T cells in the ratio of 0.3:1 for 48 hours in the presence of 5 µg/ml plate-bound anti-CD3 antibody. Cells and culture supernatants were harvested after 48 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours while cytokines were measured by ELISA. Each dot represents a mean of 5 replicates for one donor for proliferation (A) whereas each dot represents a mean of triplicate measurements of one donor for cytokines (B-D). The Wilcoxon signed rank test was used to compare co-culture of mock-treated monocytes or BiP-treated monocytes with responder T cells and ns indicates p value >0.05.

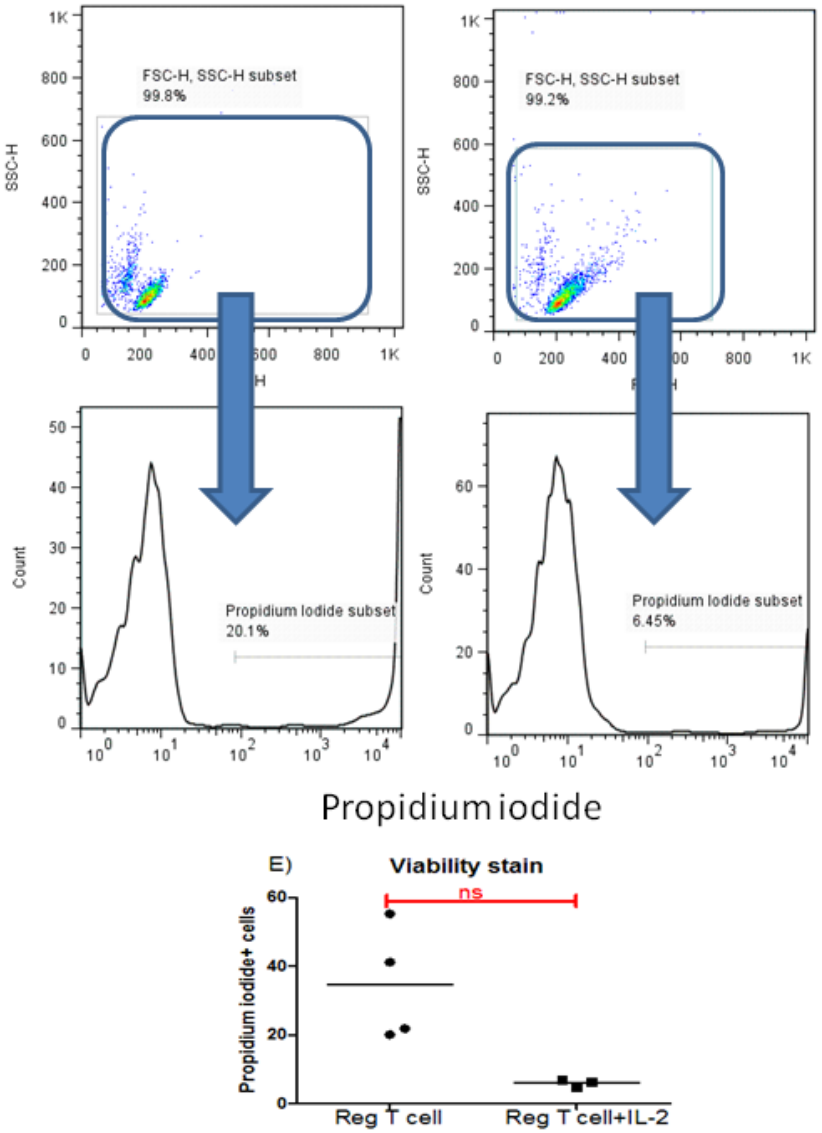
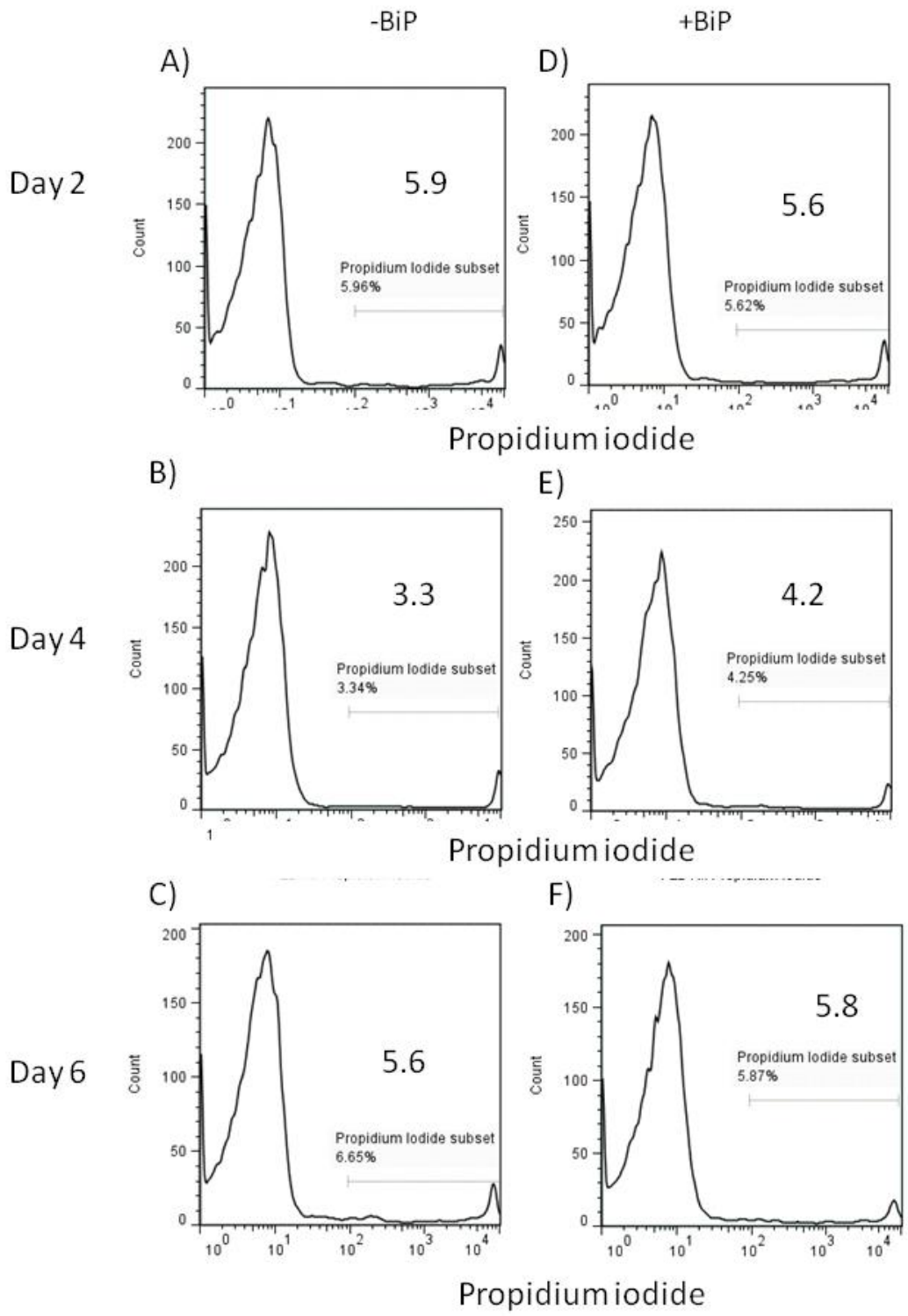


Figure 4.26 Regulatory T cells must be cultured in the presence of IL-2 to ensure that they remain viable. This experiment was performed on cone bloods and regulatory T cell isolation was performed as shown in Figure 4.1. Regulatory T cells were cultured in the presence or absence of 100U/ml IL-2 for 4 days and cells were harvested and stained with propidium iodide. Data was acquired using FACS Calibur and analysed with FlowJo. Panel (A) shows Forward v Side scatter of regulatory T cells cultured without 100 U/ml IL-2 and and (B) shows histogram of propidium iodide while (C&D) demonstrate regulatory T cells cultured in the presence of I100 U/ml IL-2. Each dot in (E) represents one donor and the Mann Whitney test was used to compare propidium iodide staining of regulatory T cells in the absence or presence of IL-2 and ns indicates p value >0.05.


-BiP
+BiP
A)
D)
Day 2
5.9
Propidium Iodide subset
5.96%
5.6
Propidium Iodide subset
5.62%
Count
Propidium iodide
B)
E)
Day 4
3.3
Propidium Iodide subset
3.34%
4.2
Propidium Iodide subset
4.25%
Count
Propidium iodide
C)
F)
Day 6
5.6
Propidium Iodide subset
6.65%
5.8
Propidium Iodide subset
5.87%
Count
Propidium iodide

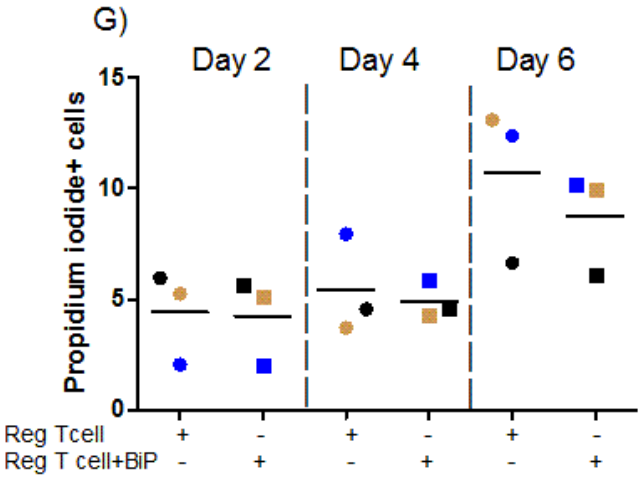
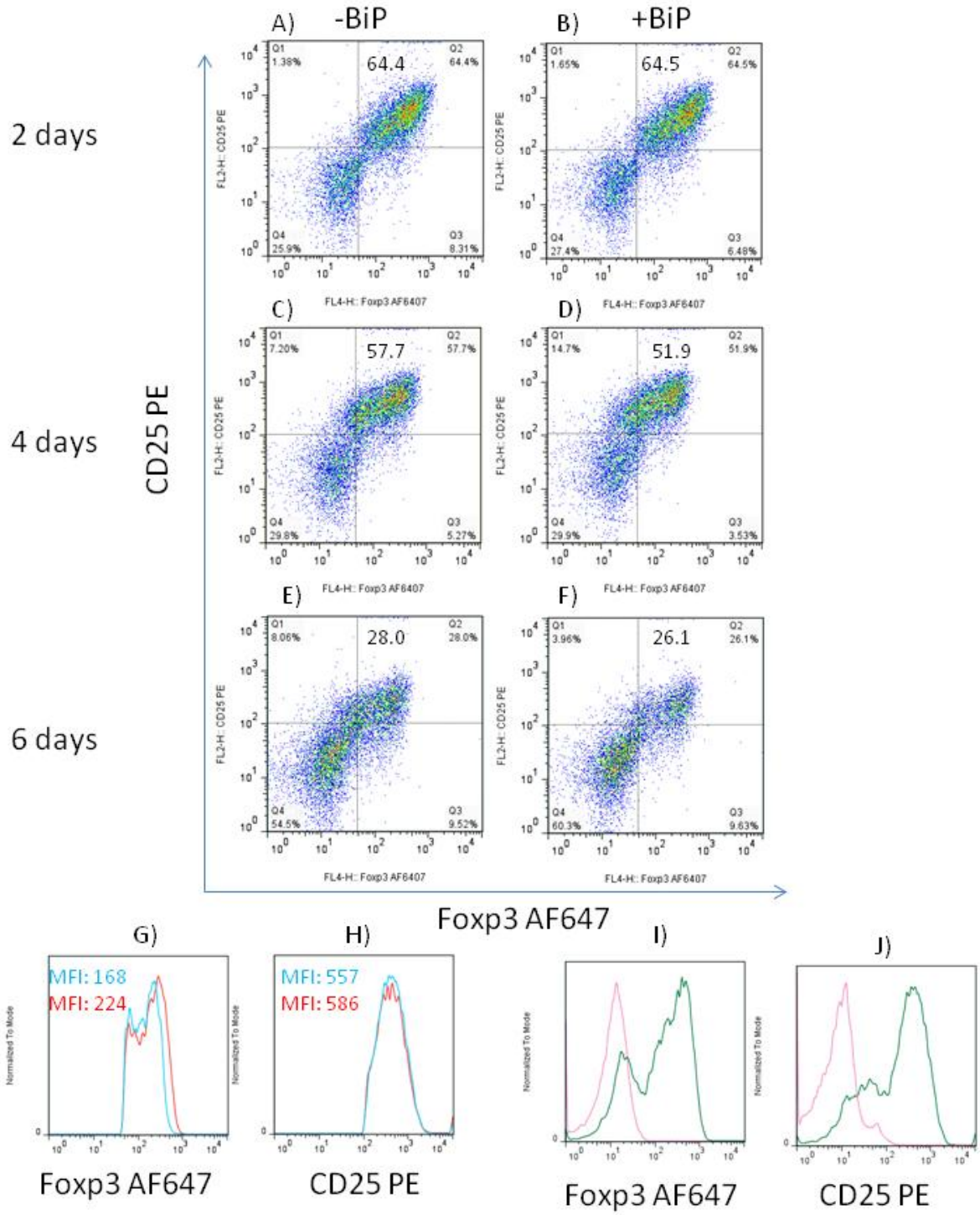


Figure 4.27 Increased cell death after 6 days of regulatory T cell in culture with the presence of IL-2. This experiment was performed on fresh bloods and regulatory T cell isolation was performed as described in Figure 4.1. Regulatory T cells were cultured in the presence of 100U/ml IL-2 with or without 10 ng/ml BiP for 2,4 and 6 days and cells were harvested and stained with propidium iodide. Data was acquired using FACS Calibur and analysed with FlowJo. Panel (A-C) contain histograms describing propidium iodide in regulatory T cells cultured without BiP while (D-F) show regulatory T cells cultured with BiP. Each dot in (G) represents one donor and a matching colour indicates the same donor.


A) -BiP
B) +BiP
C)
D)
E)
F)
2 days
4 days
6 days
CD25 PE
Foxp3 AF647
64.4
64.5
57.7
51.9
28.0
26.1
G)
H)
I)
J)
MFI: 168
MFI: 224
MFI: 557
MFI: 586
Foxp3 AF647
CD25 PE
Foxp3 AF647
CD25 PE

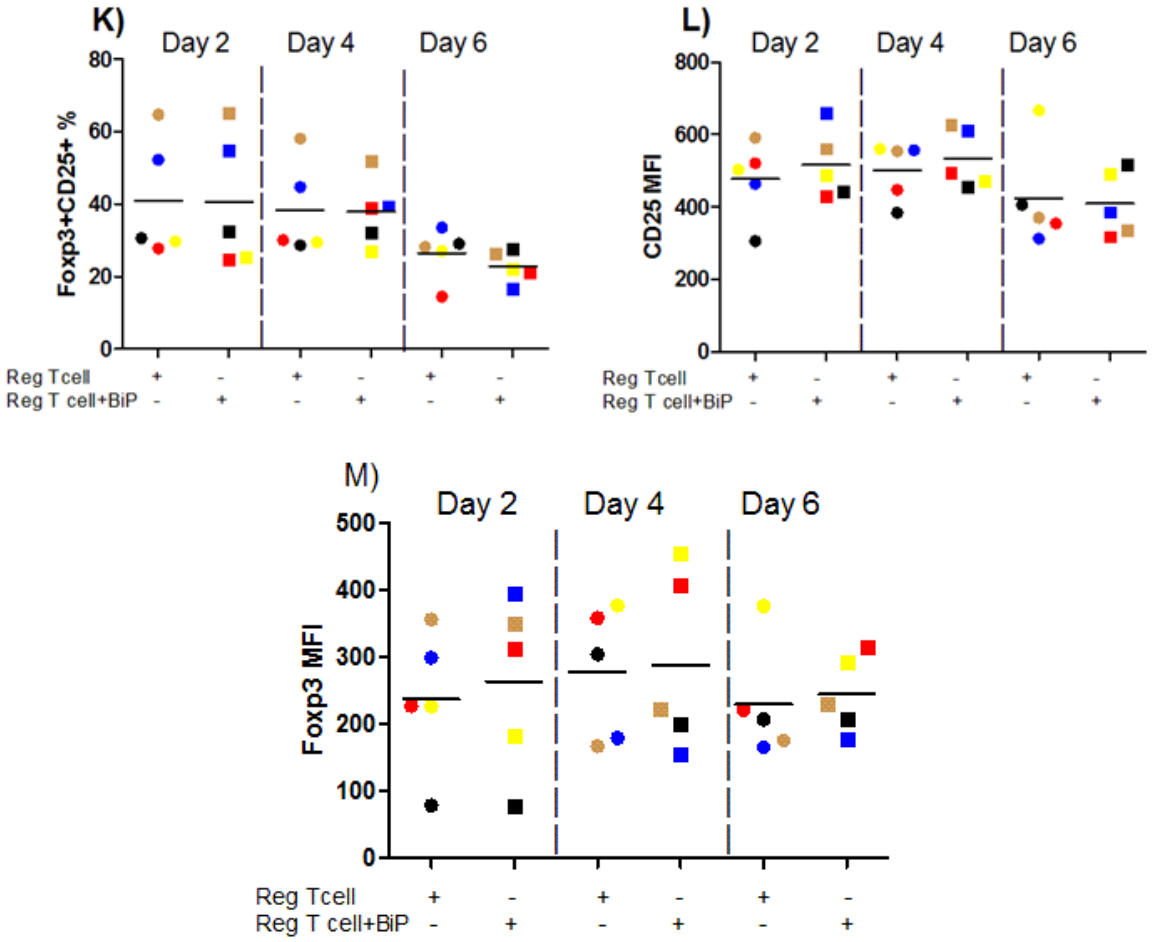


Figure 4.28 Foxp3 and CD25 expression in regulatory T cells were increased with BiP after 4 days in culture although the differences are statistically insignificant. This experiment was performed on fresh bloods and regulatory T cell isolation was performed as shown in Figure 4.1. Regulatory T cells were cultured in RPMI media supplemented with 10% FCS for 2,4 and 6 days in the presence of 100 U/ml IL-2 with or without 10 ng/ml of BiP. Surface staining of CD25 was performed followed by intracellular staining of Foxp3 using kit from BioLegend. Data was acquired on FACS Calibur and analsyed using FlowJo. Panel (A-C) show a dot plot of CD25 versus Foxp3 of regulatory T cells cultured without BiP while (D-F) show dot plots of regulatory T cells cultured with BiP. Panel (G-H) show histograms of Foxp3 (G) and CD25 (H). The blue line represents regulatory T cells cultured without BiP while the red line represents regulatory T cells cultured with BiP. Panel (I-J) contain histogram of Foxp3 (I) and CD25 (J) comparing isotype control (pink line) and antibody staining (dark green line). The remaining figures show the frequency of Foxp3+CD25+ (K), MFI of Foxp3 (L) and MFI of CD25 (M). Each dot represents one donor and a matching donor indicates a same donor.

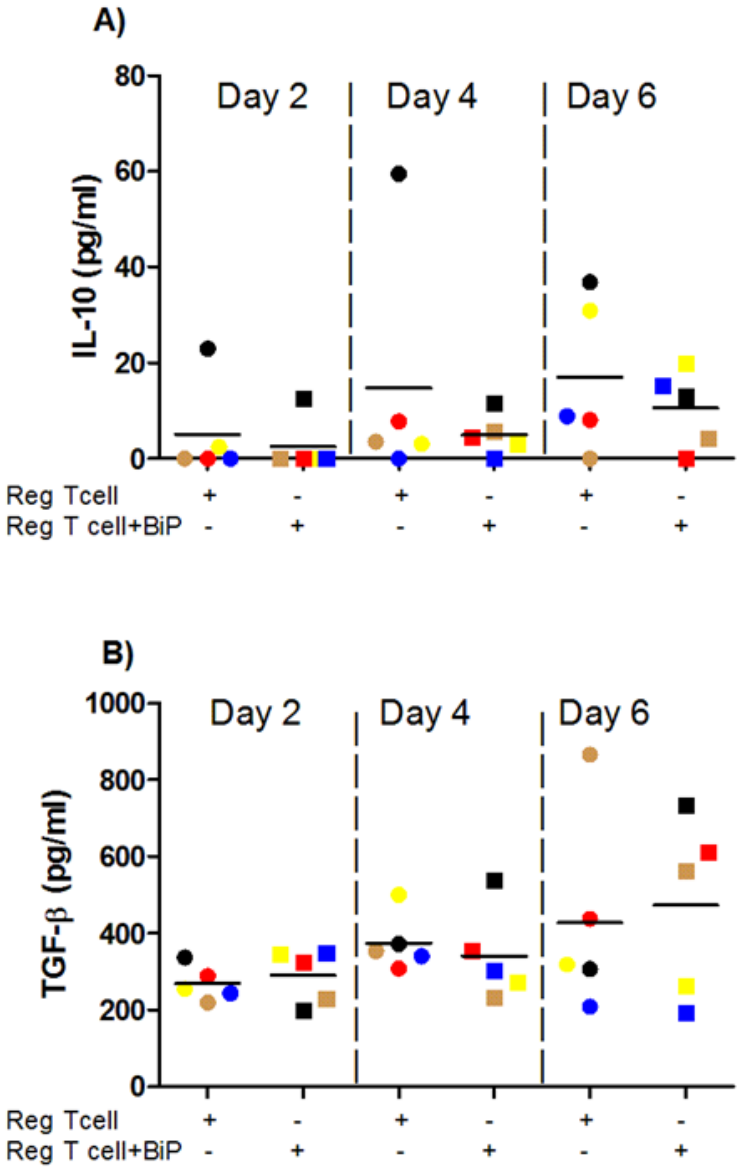
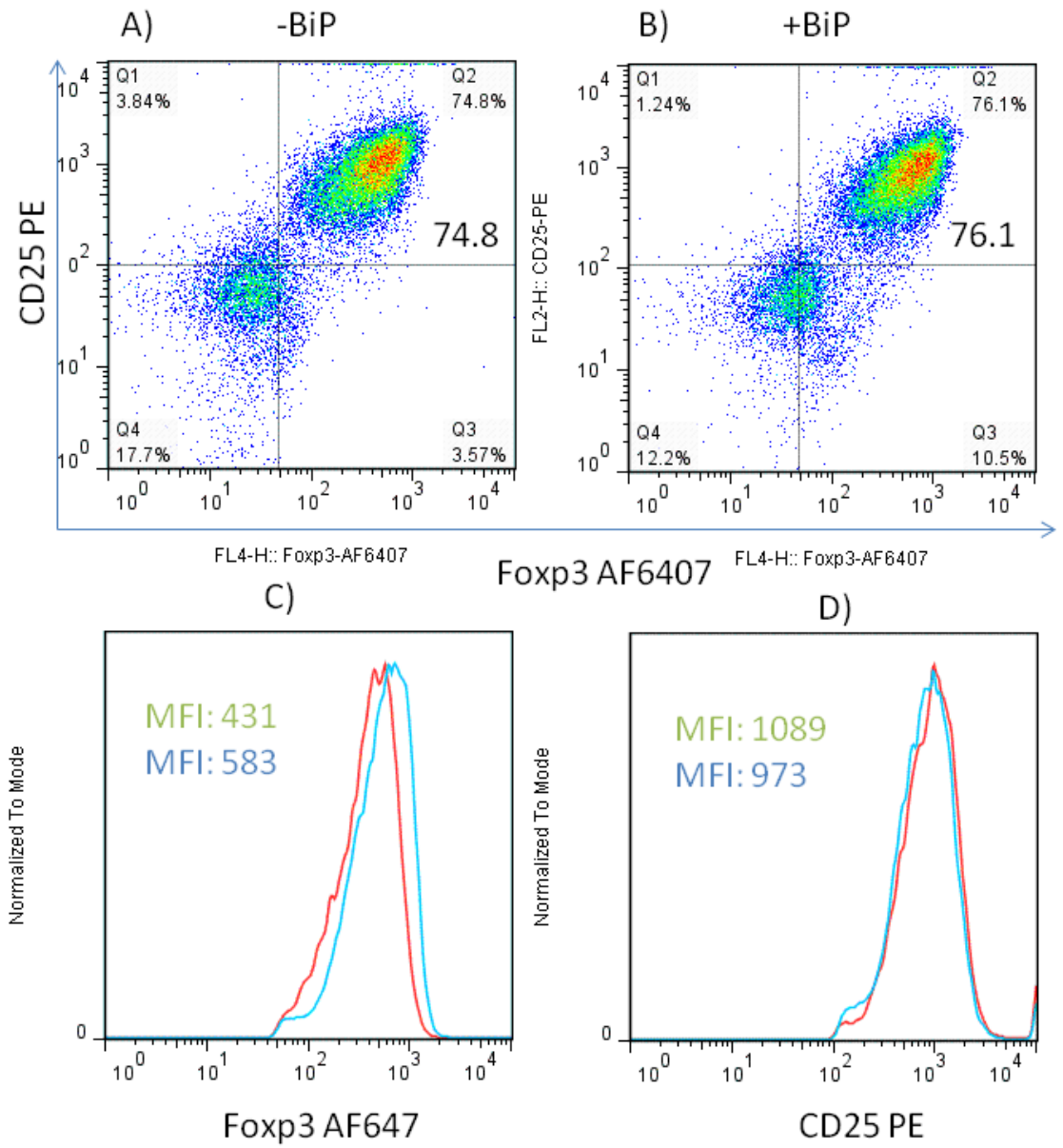


Figure 4.29 BiP reduced IL-10 from regulatory T cells in culture while the effect of BiP on TGF-β secretion is variable. This experiment was performed on fresh bloods and regulatory T cell isolation was performed as shown in Figure 4.1. Regulatory T cells were cultured in RPMI media supplemented with 10% FCS for 2,4 and 6 days in the presence of 100 U/ml IL-2 with or without 10 ng/ml of BiP. Culture supernatants were harvested and IL-10 and TGF-β levels were measured using ELISA. Panel (A) shows IL-10 concentration while (B) shows TGF-β levels from supernatant of regulatory T cell cultured in the presence of 100 U/ml IL-2. Each dot in (A-B) represents a mean of triplicate measurements of one donor and a matching colour indicates a same donor.


A)
-BiP
B)
+BiP
CD25 PE
FL2-H:: CD25-PE
Q1
3.84%
Q2
74.8%
74.8
Q4
17.7%
Q3
3.57%
Q1
1.24%
Q2
76.1%
76.1
Q4
12.2%
Q3
10.5%
FL4-H:: Foxp3-AF6407
FL4-H:: Foxp3-AF6407
Foxp3 AF6407
C)
D)
MFI: 431
MFI: 583
MFI: 1089
MFI: 973
Normalized To Mode
Normalized To Mode
Foxp3 AF647
CD25 PE

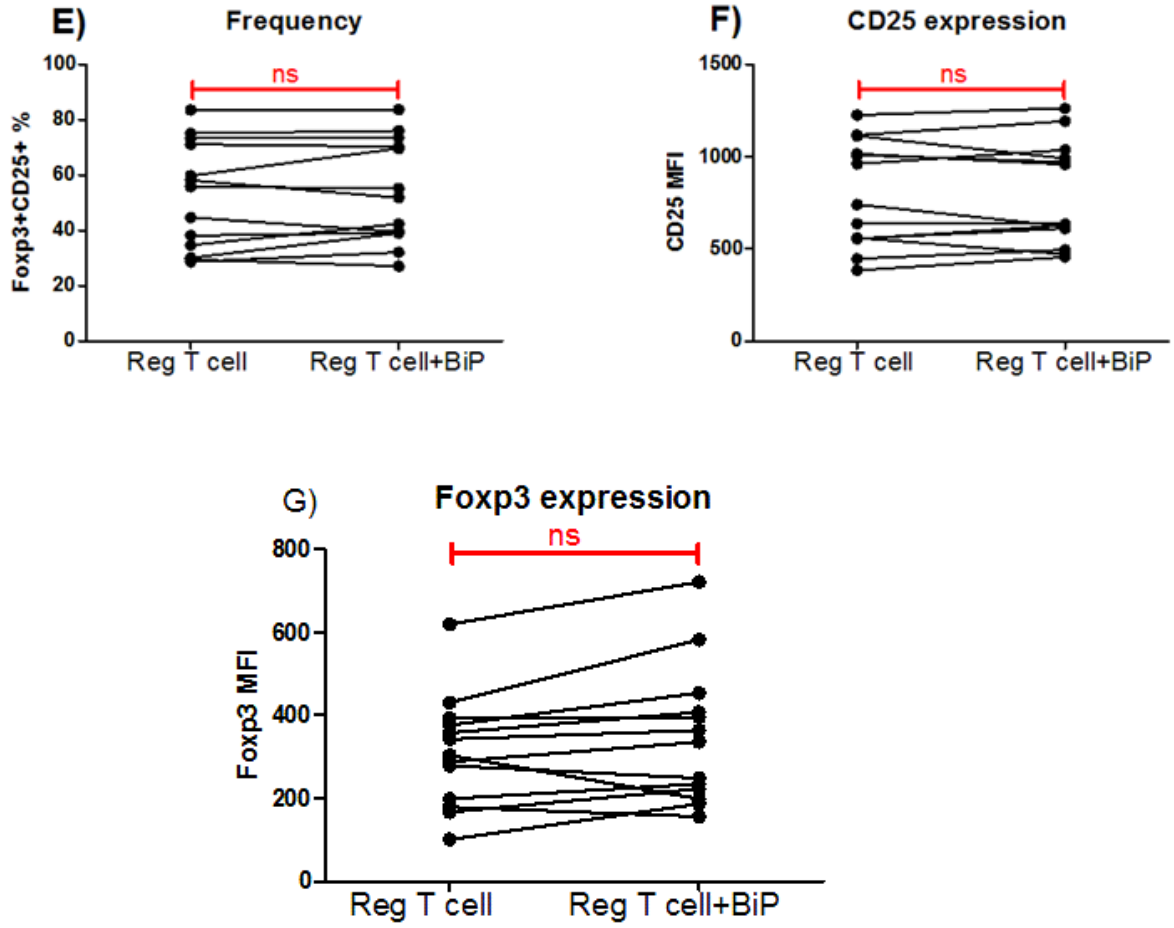


Figure 4.30 BiP may increase Foxp3 expression although the increase is statistically insignificant (p=0.06). This experiment was performed with both fresh (1 MS column) and cone bloods (2 MS columns). Regulatory T cell isolation was performed as described in Figure 4.1. Regulatory T cells were cultured in RPMI media supplemented with 10% FCS for 96 hours in the presence of 100 U/ml IL-2 with or without 10 ng/ml of BiP. Surface staining of CD25 was performed followed by intracellular staining of Foxp3 using kit from BioLegend. Data was acquired on FACS Calibur and analysed using FlowJo. Panel (A-B) show a Foxp3 v CD25 plot from lymphocyte gate while (C-D) shows histograms of Foxp3 (C) and CD25 (D). The red line represents regulatory T cells cultured without BiP while blue line represents regulatory T cells cultured with BiP. Panel (E) the shows percentage of Foxp3+CD25+ in the presence or absence of 10 ng/ml of BiP while (F) demonstrates MFI of CD25 expression and (I) shows MFI of Foxp3 expression. Each dot in (E-G) represents one of multiple donors. The Wilcoxon signed rank test was used to compare between regulatory T cells cultured with or without BiP and ns indicates p value is >0.05.

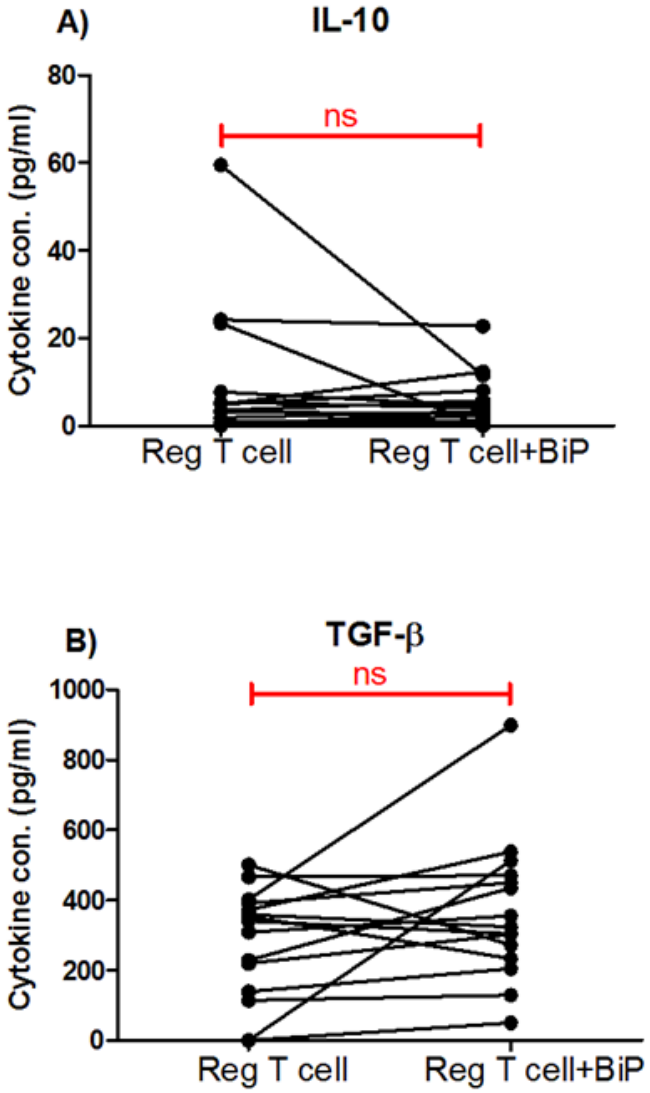
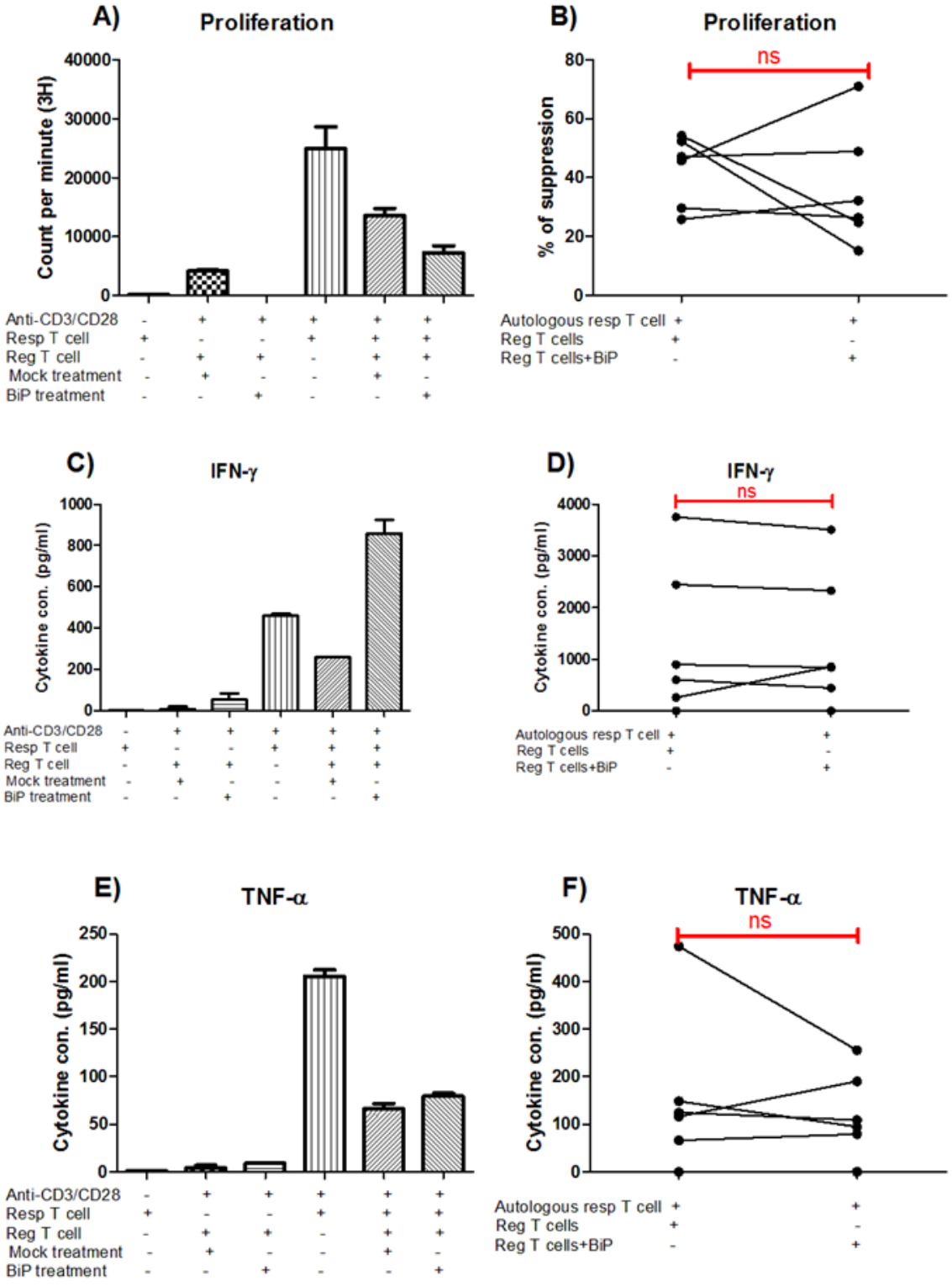


Figure 4.31 BiP may increase TGF-β secretion in regulatory T cells although the difference is statistically insignificant (p=0.09). This experiment was performed on fresh bloods. Regulatory T cell isolation was performed as shown in Figure 4.1. Regulatory T cells were cultured in RPMI media supplemented with 10% FCS for 96 hours in the presence of 100 U/ml IL-2 with or without 10 ng/ml of BiP. Culture supernatants were harvested after 96 hours and IL-10 and TGF-β levels were measured using ELISA. Each dot in (A-B) represents a mean of triplicate measurements of one donor. Panel (A) shows IL-10 concentration while (B) shows TGF-β levels from supernatant of regulatory T cell cultured in the presence of 100 U/ml IL-2. The Wilcoxon signed rank test was used to compare between regulatory T cells cultured with or without BiP and ns indicates p value is >0.05.


A)
Proliferation
Count per minute (3H)
40000
30000
20000
10000
0
Anti-CD3/CD28
Resp T cell
Reg T cell
Mock treatment
BiP treatment
B)
Proliferation
ns
% of suppression
80
60
40
20
0
Autologous resp T cell
Reg T cells
Reg T cells+BiP
C)
IFN-γ
Cytokine con. (pg/ml)
1000
800
600
400
200
0
Anti-CD3/CD28
Resp T cell
Reg T cell
Mock treatment
BiP treatment
D)
IFN-γ
ns
Cytokine con. (pg/ml)
4000
3000
2000
1000
0
Autologous resp T cell
Reg T cells
Reg T cells+BiP
E)
TNF-α
Cytokine con. (pg/ml)
250
200
150
100
50
0
Anti-CD3/CD28
Resp T cell
Reg T cell
Mock treatment
BiP treatment
F)
TNF-α
ns
Cytokine con. (pg/ml)
500
400
300
200
100
0
Autologous resp T cell
Reg T cells
Reg T cells+BiP

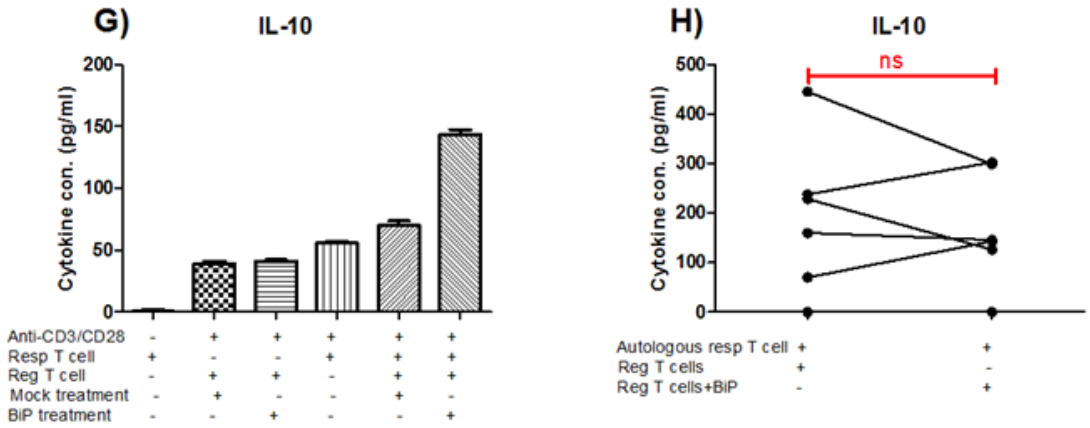


Figure 4.32 BiP-treatment on regulatory T cells for 4 days do not enhance their function. This experiment was performed on fresh blood and responder and regulatory T cell (using 1 MS column). Isolation was performed as shown in Figure 4.1. Responder T cells were cultured in RPMI media supplemented with 10% FCS for 96 hours while regulatory T cells were pre-treated with 10 ng/ml of BiP for 4 days before being washed away twice, recounted and resuspended in new media. Regulatory and responder T cells were co-cultured in a ratio of 0.3 to 1 in the presence of 1 anti-CD3/CD28 bead to 5 cells. Cells and culture supernatants were harvested after 48 hours and proliferation was measured by tritiated thymidine incorporation for 16-18 hours while cytokines were measured by ELISA. Panel (A) shows the proliferation of one donor and each bar represents a mean of 5 replicates and standard deviation as the error bar while (B) demonstrates a mean percentage of suppression in multiple donors as each dot represents one donor. Each bar in panel (IFN-γ (C), TNF-α (E), IL-10 (G)) show a mean of triplicate measurements from culture supernatants of one donor while each dot in Panel (IFN-γ (D), TNF-α (F), IL-10 (H)) illustrates a mean of triplicate measurements of one donor out of 6 donors. The Wilcoxon signed rank test was used to compare between mock treatment and BiP treatment and ns indicates p value >0.05.

4.4 Discussion

In vitro suppression assays are now widely used to determine the suppressive capacity of regulatory T cells. Before suppression assay can be performed, regulatory and responder T cells were isolated using magnetic beads. After that, the purity of regulatory and responder T cells were examined. As shown in Figure 4.3, responder T cells defined as CD4+CD25^{Low}CD127^{High} population achieved the mean purity of 92% while regulatory T cells isolated from 2 MS columns achieved the mean purity of 95% as regulatory T cells are defined as CD4+CD25^{High}CD127^{Low} population as illustrated in Figure 4.4. To further confirm these two populations, intracellular Foxp3 staining was performed. As demonstrated in Figure 4.5, 82.3% of regulatory T cells were Foxp3+ cells while 11.4% of responder T cells were Foxp3+ cells. As Foxp3 protein can be expressed by responder T cells especially after activation of responder T cells (Allan et al, 2007, Wang et al, 2007, McMurchy et al, 2013), it is important to validate that regulatory T cells isolated are truly regulatory T cells as regulatory T cells can express high levels of Foxp3. Furthermore, regulatory T cells isolated using magnetic beads can suppress the proliferation of responder T cells and cannot proliferate in vitro as demonstrated throughout this chapter. This evidence further supports that regulatory T cells isolated using this method were true regulatory T cells as Hombach et al (2007) demonstrated that human regulatory T cells cannot proliferate in *in vitro* culture without any IL-2 stimulation.

Before investigating the effect of BiP on regulatory T cell function, it was pivotal to reproduce the findings of Zanin-Zhorov and colleagues (2006) as they demonstrated that HSP60 pre-treatment on regulatory T cells can enhance their suppressive ability on responder T cell proliferation and reduce pro-inflammatory cytokines namely TNF-α and IFN-γ in the co-culture. Figure 4.7 shows that no phenotypic change on regulatory T cells pre-treated with HSP60 compared to mock treated regulatory T cells and Figure 4.8 demonstrates that HSP60 pre-treatment of regulatory T cells had no effect on the ability of regulatory T cells to suppress responder T cell proliferation but can modulate their ability to suppress IFN-γ levels in the co-culture. Zanin-Zhorov et al (2006) utilised 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay, a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye, MTT, to its insoluble formazan, giving a purple colour to represent the responder T cell proliferation,

which is a more sensitive measurement compared to measuring responder T cell proliferation by uptake of tritiated thymidine which detects newly synthesized DNA in the entire cell population. Therefore, the different method used to measure responder T cell proliferation may explain the discrepancy between the results in Figure 4.8 and Zanin-Zhorov et al (2006). This observation suggests that HSP60 may not induce phenotypic change but can modulate the function of regulatory T cells by the downregulation of IFN-γ in the co-culture.

Then, the role of BiP on regulatory T cells was investigated. Two hours of BiP pre-treatment was not enough to induce any changes in gene induction and transcription thus no change was observed in the phenotype and function of regulatory T cells cultured with BiP in various experimental settings (shown in Figure 4.14-4.17). This is in contrast with HSP60 and HSP70 where Zanin-Zhorov et al (2006) and Wachstein et al (2012) showed that two hours of HSP pre-treatment can enhance the ability of regulatory T cells to suppress responder T cell proliferation. Furthermore, both HSPs had been shown to modulate regulatory T cell function via TLR2. Therefore, it is highly unlikely that BiP binds to TLR2 on immune cells to mediate biological effects as shown in Figure 4.15. Since BiP has been shown to modulate monocyte function (Corrigall et al, 2004), co-cultures of BiP-treated monocytes and regulatory T cells were performed and the suppressive capacity of regulatory T cells on responder T cells was examined after the addition of responder T cells. As demonstrated in Figure 4.24, regulatory T cell suppressive function was not influenced by the presence of BiP-treated monocytes but BiP-treated monocytes can reduce the proliferation of responder T cells (shown in Figure 4.25). In addition, BiP can directly modulate responder T cell function by reducing their proliferation in the presence of anti-CD3/CD28 beads; but no change in cytokine secretion profile as demonstrated in Figure 4.15 and 4.16.

Clearly, data from responder T cells showed that BiP may take longer time to modulate responder T cell function hence regulatory T cells were cultured with BiP for a period of longer than 2 hours. The kinetic of BiP treatment on regulatory T cells were performed and examined (demonstrated in Figure 4.27-4.29) by several readouts. Any BiP effect on regulatory T cells were investigated using Foxp3 and CD25 expression and IL-10 and TGF-β cytokine secretions from regulatory T cells. Based on the kinetic experiments,

regulatory T cells were cultured with BiP for 4 days. Figure 4.30 illustrates that regulatory T cells cultured with BiP may increase their Foxp3 expression although the increase is statistically insignificant (p=0.06). The importance of Foxp3 in regulatory T cells was highlighted by Ferraro and colleagues (2014) by investigating the heterogeneity of human regulatory T cells. Foxp3 and other genes associated with regulatory T cells were examined from peripheral blood of healthy donors and they observed that Foxp3 gene expression was tightly clustered between individuals showing the importance of this gene to regulatory T cells. In addition, Wan and Flavell (2007) demonstrated that reduction of Foxp3 expression in murine regulatory T cells lead to impaired suppressive function and altered surface properties. These mice also developed an aggressive autoimmune syndrome. Therefore, a small increase of Foxp3 expression in regulatory T cells may lead to enhanced suppressive capacity. Again the same trend was observed with TGF-β secretion. Figure 4.31 shows that TGF-β secretion was increased although the increase is statistically insignificant (p=0.09) However, since regulatory T cells used for these experiments were isolated from 1 MS column, it is possible that these cells were contaminated with high frequency of responder T cells and monocytes. In conclusion, 4 days of BiP treatment of regulatory T cells may enhance Foxp3 expression and induce TGF-β secretion.

The main question now is whether these changes induced by BiP on regulatory T cells are translated to enhanced suppression of regulatory T cells on responder T cells. However, these changes do not modulate the ability of regulatory T cells to further enhance their suppression of responder T cells or cytokine levels in the co-culture (shown in Figure 4.32). This can be partly explained by the fact that *in vitro* suppression assays do not require TGF-β to suppress responder T cells. Conflicting evidence has been reported in the literature. Oberle et al (2007) and Annunziato et al (2002) demonstrated that TGF-β plays a partial role in the suppression of human regulatory T cells towards responder T cells, by using blocking antibodies. In contrast, Jonuleit et al (2001) and Dieckmann et al (2001) argued otherwise using the same strategy. An elegant experiment was performed by Ferraro et al (2014) showing the correlation between expression of different genes associated with human regulatory T cells and the ability of regulatory T cells to suppress proliferation of responder T cells. The only gene that highly correlates with suppressive capacity of regulatory T cells is T cell immunoglobulin and ITIM domain (TIGIT), a surface

protein expressed on memory, activated and regulatory T cells, but not the TGF-β gene. In addition, it is possible that TGF-β secretion of regulatory T cells may enhance the suppressive capacity of regulatory T cells on the proliferation of responder T cells in the presence of dendritic cells or monocytes in the co-cultures because the co-culture here was performed in the presence of anti-CD3/CD28 beads only.

A small increase in Foxp3 expression and TGF-β secretion by regulatory T cells induced by BiP did not lead to better suppressive capacity of regulatory T cells on responder T cells. However, TGF-β is a cytokine with pleiotropic effect: it can induce various biological effects on other immune cells. It is well established that TGF-β is an important cytokine in inducing the development of regulatory T cells. Zheng et al (2002), Fantini et al (2004) and Wang et al (2009) demonstrated the role played by TGF-β in inducing regulatory T cell development. Zheng et al (2002) demonstrated that TGF-β and a low-dose antigen can induce human CD4+CD25- to become Th3 suppressor cells, an induced regulatory T cell characterised by the presence of Foxp3, and they can secrete TGF-β while Wang et al (2009) showed that TGF-β and retinoic acid can convert naïve human CD4+ T cells into Foxp3+ regulatory T cells with stable Foxp3 expression and potent suppressive capability. Furthermore, TGF-β can induce the upregulation of CD73 molecule on murine CD4+ T cells as demonstrated by Regateiro et al (2011). CD73 is an important ectoenzyme that will catalyse the conversion of adenosine monophosphate (AMP) into adenosine, which in turn will help to suppress CD4+ T cell proliferation. In addition, TGF-β can exert an immunosuppressive effect on monocytes, B cells and dendritic cells. Musso et al (1990) reported that TGF-β can downregulate IL-1 secretion from human monocytes induced by IL-6. TGF-β can modulate human B cell function by directing the immunoglobulin (Ig) A switching process as demonstrated by van Vlasselaer and co-workers (1992). Furthermore, Pallotta and colleagues (2011) indicates that TGF-β treatment on murine plasmacytoid dendritic cells (pDC) can induce an increased expression of IDO, an immunomodulatory enzyme important in suppressing responder T cell proliferation. Most importantly, Esquerre and colleagues (2008) showed that human regulatory T cells can inhibit the polarisation of T helper cells toward APCs via a TGF-β dependent mechanism. TGF-β secreted from regulatory T cells interfered with the polarisation of the T helper secretory machinery

toward APC thus affecting antigen delivery from APC to T helper cells so no polarisation of T helper cell takes place.

BiP-induced changes to regulatory T cells pose another question about how BiP may signal to mediate these effects. Corrigall et al (2003) reported that BiP has a specific receptor that is expressed by more than 95% in human monocytes, up to 50% in B cells and 10% in T cells. However, the identity of this receptor remains elusive. Therefore, we can only speculate about the signalling pathways involved in mediating a small increase in Foxp3 expression and TGF-β secretion upon BiP ligation with this unknown receptor on regulatory T cells. There are several key signalling pathways that might be involved in the upregulation of Foxp3, namely: TCR signalling pathway, PI3-Akt-mammalian target of rapamycin (mTOR) pathway, NF-kB-CRel pathway or IL-2-signal transduction and transcription 5 (STAT5) axis. These pathways were listed by Yuan and Malek (2012) as key signalling pathways involved in the development of regulatory T cells. However, STAT5 is probably the most important adaptor molecule involved in the upregulation of Foxp3 as indicated by Yuan and Malek (2012) because of its location. STAT5 can bind to the Foxp3 promoter and at the same time it can also bind to the enhancer region (CNS2) of Foxp3 gene to stabilise Foxp3 expression in regulatory T cells. Most importantly, the pivotal role played by STAT5 can be demonstrated through an animal model. Burchill et al (2007) showed that STAT5 deletion in T cells can prevent regulatory T cell development in mice and they demonstrated STAT5 binding to Foxp3 promoter promotes regulatory T cell development.

Another effect of BiP on regulatory T cells is an increase of TGF-β secretion although the increase is statistically insignificant. The signalling pathways involving TGF-β signalling via its receptor has been the focus of most of the research in TGF-β downstream signalling. Using Jurkat cells to study TGF-β regulation in response to apoptotic cells, Xiao et al (2008) demonstrated the involvement of p38 MAPK, ERK and JNK in TGF-β transcription, whereas translation required activation of Rho GTPase, PI3K, Akt, and mTOR. One of the most important findings drawn from their work was that the induction of TGF-β in response to apoptotic cells used different induction pathways compared to cells stimulated with either phorbol myristate (PMA) or LPS. To add to the complexity of TGF-β induction, Furler and Uittenbogaart (2012) reported that the transcription factor GLI2 is important in regulating TGF-β expression in human CD4+ T cells. They proposed that GLI2 can bind to TGF-β

promoter and the knockdown of GLI2 in human regulatory T cells significantly decreased TGF-β transcription. Therefore, further investigation into the signalling pathways involved in increased Foxp3 expression and increased TGF-β will be conducted in the future to confirm the effect of BiP pre-treatment on regulatory T cells. In conclusion, BiP may induce immunoregulatory phenotypes in regulatory T cells after 4 days of treatment.

These results may add further support to the hypothesis proposed by Shields et al (2012), suggesting BiP as a member of resolution associated molecular patterns (RAMP). RAMPs are defined as 'highly evolutionarily conserved, multi-functional, constitutively expressed proteins whose immunoregulatory activity is dependent upon their rapid decompartmentalization from the intracellular environment either actively, following cell stress, or passively, via necrotic cell death' (Shields et al, 2012). It is suggested that RAMP is involved in the resolution stage, the final stage of immunological response; therefore BiP may be considered a RAMP by further potentiating an immunregulatory phenotype in regulatory T cells. Another interesting observation from this chapter is that BiP can directly reduce responder T cell proliferation in the presence of co-stimulation or indirectly via BiP treated monocytes but does not affect the cytokine profile of these cells. This raises another important question about what changes BiP makes to these cells to cause them to reduce their proliferative capacity compared to responder T cells cultured with media alone. This question will be addressed and investigated in the next chapter.

4.5 Conclusion

The conclusions of this chapter are as follows:-

- Two hours of BiP pre-treatment does not enhance regulatory T cell phenotype or function either in the presence of anti-CD3 antibody, anti-CD3/CD28 beads or monocytes
- Four days of BiP pre-treatment may increase Foxp3 expression and TGF-β secretion from regulatory T cells although the differences are statistically insignificant but does not enhance the suppressive capacity of regulatory T cells
- BiP can indirectly reduce responder T cell proliferation via BiP treated monocytes
- BiP can reduce responder T cell proliferation in the presence of anti-CD3/CD28 beads after 4 days in culture

Chapter 5

The effect of soluble BiP on responder T cell phenotype and function

5 The effect of soluble BiP on responder T cell phenotype and function

5.1 Introduction

Soluble BiP may modulate regulatory T cell phenotype by an increase in Foxp3 expression and TGF-β secretion in the previous chapter. However, at least in *in vitro* suppression assay these changes did not appear to enhance their regulatory activity. In addition, responder T cells cultured with BiP for 4 days in the presence of anti-CD3/CD28 beads reduced their proliferation compared to responder T cells cultured without BiP. The effect of BiP on responder T cells was observed in cone blood. Since responder T cells can be easily isolated in large numbers, these experiments were repeated using responder T cells from fresh blood.

The aim of this chapter is to determine how soluble BiP can affect responder T cell function by reducing proliferation in the presence of TCR stimulation and co-stimulation in the form of anti-CD3/CD28 beads. Although proliferation was reduced, there was no change observed with the cytokine profile (IFN-γ, TNF-α and IL-10) from supernatants of the cultures. One plausible explanation of this observation is that BiP can induce regulatory T cell development within responder T cells thus reducing the proliferation of the remaining responder T cells. Induction of regulatory T cells from responder T cells indirectly by BiP was reported by Corrigall and colleagues (2009) via monocyte-derived dendritic cells. They observed that human monocyte-derived dendritic cells cultured with BiP can induce regulatory T cell development, which is characterised by an increase in the frequency of CTLA-4 positive cells but no change was observed in the frequency of Foxp3 positive cells. These induced regulatory T cells can suppress proliferation of autologous responder T cells. This work implies that BiP may induce TR1 regulatory T cells, which are characterised by the absence of Foxp3 but secrete IL-10. In addition, immunisation of mice with BiP has been shown to induce regulatory T cell development. Brownlie and colleagues (2006) demonstrated that BiP can induce IL-4 and IL-10 secreting regulatory T cells when mice were immunised with BiP while Foo (2011) reported that BiP can induce antigen-specific regulatory T cells. These cells have the ability to suppress the recall response of type II collagen (CII)-reactive cells in *in vitro* suppression assays. These CD4+CD25+Foxp3-PD-1+ cells can secrete IL-10, TGF-β and soluble CTLA-4, a novel splice variant of CTLA-4.

Furthermore, HSP60 has been shown to convert human CD4+CD25- responder T cells into CD4+CD25+Foxp3+ regulatory T cells (de Kleer et al, 2010). A study by van Herwijnen and co-workers (2012) showed that the HSP70 epitope can induce CD4+CD25+Foxp3+ which can suppress proteoglycan-induced arthritis in mice.

5.2 Objectives

The objectives of this chapter are therefore as follows:-

- To confirm the effect of BiP in reducing responder T cell proliferation using cells from fresh blood rather than cone blood
- To examine the potency of BiP on responder T cell proliferation by titrating of BiP effect on responder T cell proliferation
- To examine the effects of BiP on responder T cell phenotype
- To investigate whether any phenotypic change after BiP treatment can lead to a change in function

5.3 Results

5.3.1 BiP can reduce responder T cell proliferation in a dose dependent manner

As previously shown in Figure 4.18(B) and confirmed here in Figure 5.1, responder T cells isolated from cone bloods can reduce their proliferation in the presence of anti-CD3/CD28 beads with 10 ng/ml of BiP in culture. Responder T cells can be easily isolated with a high yield from fresh blood; therefore a dose-titration of BiP was performed on responder T cells isolated from fresh blood. Figure 5.2(A) shows that responder T cell proliferation was reduced in a dose-dependent manner when cultured with BiP in the presence of anti-CD3/CD28 as indicated by the mean proliferation in 6 donors even though a significant difference was observed only when comparing 0 and 0.001 ng/ml ($p=0.03$) and 0.1 ng/ml ($p=0.03$). Furthermore, responder T cells cultured with BiP did not induce any changes to the cytokines measured from the culture as demonstrated in Figure 5.2 (B-D). IFN-γ (B) and IL-10 (D) levels from responder T cells cultured with BiP were quite constant among multiple donors while there is a huge variability of TNF-α levels (C) reported from multiple donors.

5.3.2 Cell death does not account for the reduced proliferation induced by BiP

To confirm that BiP can suppress responder T cell proliferation and this was not due to cell death, propidium iodide staining was performed. As illustrated in Figure 5.3, responder T cells cultured with BiP (Panel A) had a slightly lower percentage of dead cells (3.53%) compared to responder T cells cultured without BiP (5.24%) (Panel B). However, the mean percentage of dead cells between responder T cells cultured with BiP (12.8%) was slightly higher than responder T cells without BiP (11%) and the difference is not statistically significant ($p=0.125$, $n=5$) as shown in Panel C.

5.3.3 BiP can increase the frequency of Foxp3+ cells but not CTLA-4+ cells within unstimulated responder T cell populations

Corrigall et al (2009) demonstrated that BiP can induce the development of induced regulatory T cells characterised by the presence of CTLA-4 and CD27 molecules. These regulatory T cells were induced by BiP-treated dendritic cells derived from monocytes. BiP can directly reduce responder T cell proliferation; therefore it can be hypothesised that BiP may induce a proportion of responder T cells to become regulatory T cells. Therefore,

intracellular staining of CTLA-4 and Foxp3 was performed on responder T cells cultured in the presence of anti-CD3/CD28 beads with or without BiP. To confirm that the antibody staining is working, a comparison was performed between antibody staining and isotype control. Figure 4.27(I) shows that anti-Foxp3 antibody was working compared to isotype control. Therefore, a comparison between anti-CTLA-4 antibody and isotype control was performed shown in Figure 5.4. Freshly isolated responder T cells stained with anti-CTLA-4 antibody staining (orange line) overlaps with unstained sample (red line). However, isotype staining control (blue line) shows a shift to the right indicating that isotype control cannot be used to control for anti-CTLA-4 antibody staining. To control for anti-CTLA-4 staining, another strategy can be used. Fluorescence Minus One (FMO) contains all the flurochromes in a panel, except for the one that is being measured. In this co-stain experiment, anti-CTLA-4 staining was controlled by comparing co-stain of both anti-CTLA-4 and Foxp3 antibodies with anti-Foxp3 antibody alone so that CTLA-4 gating can be performed.

Figure 5.5 illustrates the frequency of Foxp3+ and CTLA-4+ cells within responder T cells cultured in the presence of anti-CD3/CD28 beads. Data from one donor show that responder T cells cultured with BiP decreased Foxp3+ frequency (5.7%, Panel C) compared to responder T cells cultured without BiP (7.23%, Panel A). However, responder T cells cultured with BiP increase CTLA-4+ frequency from 21.7% (Panel B) to 24.4% (Panel D). To confirm that antibody staining is working, a comparison between antibody staining and appropriate control was performed. As shown in Panel (E-F), anti-Foxp3 and CTLA-4 antibodies stained stimulated responder T cells (red line) compared to appropriate controls (blue line). However, the mean frequency of Foxp3+ and CTLA-4+ cells within stimulated responder T cells with BiP did not induce significant changes compared to responder T cells cultured without BiP. As shown in Panel (G-H), responder T cells cultured with BiP had 9.02% of Foxp3+ and 28.2% of CTLA-4+ cells compared to 11.02% of Foxp3+ and 25.8% of CTLA-4+ cells within stimulated responder T cells (Foxp3: p=0.07, n=7) (CTLA-4: p=0.2188, n=7).

Human responder T cells can transiently express Foxp3 (Allan et al, 2007, Wang et al, 2007, and McMurchy et al, 2013) and CTLA-4 (Tran et al, 2007) after activation. Therefore, the intracellular staining of Foxp3 and CTLA-4 was performed on responder T cells cultured in the absence of anti-CD3/CD28 beads with or without BiP. To control for Foxp3 and CTLA-4 staining, comparison between Foxp3 and CTLA-4 antibody staining was performed against

appropriate controls as shown in Figure 5.6. Data were acquired and gated on lymphocyte gate (Panel A&D) then further sub-gated based on Foxp3 expression (Panel B&E) and CTLA-4 expression (C&F). Responder T cells had more Foxp3+ cells with antibody staining (Panel B) compared to isotype control (Panel E) (2.26% versus 0.34%) while anti-CTLA-4 antibody staining (Panel C) stained more compared to FMO control (Panel F) (1.04% versus 0.20%). These data confirmed that both antibody stains are working. Using similar gating strategy as Figure 5.6, data as shown in Figure 5.7 was acquired and gated on lymphocyte gate (Panel A&D) and further sub gated on Foxp3 expression (Panel B&E) and CTLA-4 expression (Panel C&F). Foxp3 expression increased when responder T cells were cultured with BiP (2.27%, Panel E) compared to responder T cells cultured without BiP (0.41%, Panel B). Similarly, CTLA-4 expression increased as measured by the CTLA-4 frequency from 0.44% (Panel C) to 1.06% (Panel F) when cultured with BiP. However, cumulative data from multiple donors suggest that responder T cells cultured with BiP can increase Foxp3+ cells (Panel G) (0.92% versus 2.21%, p=0.0078, n=8) but not CTLA-4+ cells (Panel H) (3.08% versus 3.41%,p=0.3125, n=8) which suggests a novel effect of BiP on responder T cells.

5.3.4 BiP can increase the frequency of Foxp3+CD25+ cells and induce IL-10 secretion within unstimulated responder T cells

An increase of Foxp3+ cells in responder T cells cultured with BiP may suggest that BiP can convert responder T cells to become induced regulatory T cells. One of the most important markers used to identify regulatory T cells is the CD25 molecule. CD25 is an alpha chain of IL-2 receptor and it is constitutively expressed on regulatory T cells. Therefore, to confirm that BiP can induce regulatory T cell development from responder T cells, surface staining of CD25 and intracellular staining of Foxp3 were performed. However, as shown in Figure 4.24, BiP treated monocytes can reduce the proliferation of responder T cells after 2 days in culture. Therefore, a kinetic experiment was performed to determine the effect of BiP on inducing Foxp3+CD25+ cells within responder T cells at 3 different time points; Day 2, 4 and 6.

Before the kinetic experiment was performed, it was vital to see how long responder T cells can be cultured in media alone. Figure 5.8 illustrates that the percentage of dead cells in culture as indicated by the percentage of propidium iodide positive cells. Panel (A-C) show responder T cell culture without BiP at Day 2, 4 and 6 while responder T cells cultured

with BiP were shown in Panel (D-F). Data from Day 2 and 4 showed responder T cells cultured with BiP had slightly lower percentages of dead cells compared to responder T cells cultured without BiP (Day 2; 2.84% versus 3.33%, Day 4; 4.33% versus 5.01%). However, responder T cells cultured with BiP for 6 days had slightly higher percentages of dead cells compared to responder T cells cultured without BiP (8.41% versus 8.08%). This trend can be observed in the mean percentages of dead cells in multiple donors as shown in Panel G. Responder T cells cultured with BiP had lower propidium iodide+ cells at Day 2, 4 and 6 compared to responder T cells cultured without BiP (Day 2; 2.99% versus 3.26%, Day 4; 4.23% versus 5.04%, Day 6; 9.87% versus 10.07%). In addition, the data indicate a two fold increase of dead cells after 6 days (10%) in culture compared to 4 days (5%) in culture. After that, a co-stain of anti-Foxp3 and anti-CD25 antibody was performed on responder T cells.

To validate the antibody staining, a comparison was performed between anti-Foxp3 and anti-CD25 antibody staining (Panel A-B) with isotype control (Panel C-D) as shown in Figure 5.9. Responder T cells with anti-Foxp3 and CD25 antibody staining showed higher frequency of Foxp3+CD25+ (0.736%) compared to isotype control (0.13%). Then, the kinetic experiment was performed on responder T cells culture and a co-stain of anti-Foxp3 and anti-CD25 staining was performed (Figure 5.10). Responder T cells cultured without BiP were shown in Panel (A-C) while panel D-F show responder T cells cultured with BiP. BiP can induce an increase in Foxp3+CD25+ population within unstimulated responder T cells at all-time points (Day 2; 0.639% versus 0.27%, Day 4; 1.56% versus 1.32%, Day 6; 2.21% versus 1.89%). However, the cumulative data from multiple donors showed significant increase of Foxp3+CD25+ in responder T cells cultured with BiP at Day 2 and 4 but not at Day 6 (Panel G-J). BiP can induce higher Foxp3+CD25+ frequency (2.4%) within responder T cells compared to responder T cells cultured without BiP (1.6%) ($p=0.03$, $n=6$). This trend continues when responder T cells were cultured for 4 days. Responder T cells cultured with BiP had a significantly higher frequency of Foxp3+CD25+ compared to responder T cells cultured without BiP ($p=0.03$, $n=6$). However, no difference was observed when comparing responder T cells cultured with or without BiP at Day 6 ($p=0.6250$, $n=5$). This could be explained by the fact that a two fold increase in dead cells after 6 days in culture compared to dead cells after 4 days in culture was observed. To support the contention that BiP may

convert responder T cells into regulatory T cells, cytokines associated with regulatory T cells were measured from the culture supernatants, namely IL-10 and TGF-β.

Figure 5.11 demonstrates IL-10 and TGF-β measured from the culture supernatants of responder T cells cultured with or without BiP. As previously mentioned, the latent TGF-β1 isoform was measured in the cultures. Only 1 out of 5 donors produced IL-10 in the culture and the concentrations of IL-10 produced were very low (< 10 pg/ml) as shown in Panel A. However, IL-10 concentrations from one donor (red dot in Panel A) increased when responder T cells cultured with BiP at all-time points were compared to responder T cells cultured without BiP (Day 2; 0 pg/ml versus 2 pg/ml, Day 4; 0 pg/ml versus 2 pg/ml, Day 6; 0 pg/ml versus 7 pg/ml). However, responder T cells cultured with BiP produced lower concentrations of TGF-β compared to responder T cells cultured without BiP at Days 2 and 4 but not Day 6 as demonstrated in Panel B (Day 2; 96 pg/ml versus 117 pg/ml, Day 4; 334 pg/ml versus 404 pg/ml, Day 6; 182 pg/ml versus 151 pg/ml). Since the effect of BiP on responder T cell proliferation was observed at Day 4 and the increase of the frequency of Foxp3+CD25+ within responder T cells cultured with BiP peaked at Day 4, the effect of BiP on responder T cell phenotype and function was investigated on Day 4.

The effect of BiP on responder T cell phenotype was examined after 4 days in culture by measuring the frequency of Foxp3+CD25+ cells within responder T cells. As illustrated in Figure 5.12, responder T cells cultured with BiP had higher frequency of Foxp3+CD25+ cells compared to responder T cells cultured without BiP. One donor demonstrates a 5 fold increase in Foxp3+CD25+ cells (0.7% compared with 3.5%, Panel A&C) when cultured with BiP while another donor had a slight increase in Foxp3+CD25+ from 4.65% to 5.28% (Panel B&D) when responder T cells were cultured with BiP. The mean frequency of Foxp3+CD25+ within responder T cells cultured with BiP had significantly higher frequency of Foxp3+CD25+ cells compared to responder T cells cultured without BiP (3.69% versus 2.38%, p=0.0039, n=9) as shown in Panel E. The increase in Foxp3+CD25+ may affect the function of BiP-induced Foxp3+CD25+ cells. IL-10 and TGF-β concentrations were measured from the culture supernatants as shown in Figure 5.13. IL-10 secreted from responder T cells cultured with BiP was higher than the IL-10 secretion from responder T cells cultured without BiP (2 pg/ml versus 47 pg/ml, p=0.0039, n=9, Panel A) whereas no significant difference of TGF-β was observed between responder T cells cultured with or without BiP (440 pg/ml versus 483

pg/ml, p=0.3594, n=9, Panel B). In conclusion, BiP pre-treatment can induce an increase in the frequency Foxp3+CD25+ within responder T cells and induce secretion of IL-10 from the culture.

5.3.5 IL-2 induce an increase of Foxp3+CD25+ cells within responder T cells but the combination of BiP and IL-2 can increase the frequency of Foxp3+CD25+ and inducing IL-10 secretion within unstimulated responder T cells

IL-2 is an important survival factor for the development of human induced regulatory T cells from responder T cells as reported by Zheng et al (2004). Therefore, to confirm that BiP-induced Foxp3+CD25+ cells were regulatory T cells, IL-2 was added to the culture of responder T cells. Data from the kinetic experiment demonstrated a two fold increase in dead cells in the responder T cell culture at Day 6 (Figure 5.8(G)) and BiP can only increase Foxp3+CD25+ cell frequency at Day 2 and 4 but not Day 6 (Figure 5.10). Hence, the role of IL-2 in inducing regulatory T cells was investigated by comparing responder T cells cultured in the absence or presence of IL-2 with or without BiP for 6 days as shown in Figure 5.14. Panel A&C show responder T cells cultured without IL-2 while panel B&D demonstrate responder T cells cultured with IL-2. Responder T cells cultured with BiP for 6 days had a lower percentage of Foxp3+CD25+ cells (0.60%) compared to responder T cells cultured without BiP (0.66%) (Panel A&C). In the presence of IL-2, responder T cells showed an increase of Foxp3+CD25+ cells (3.40%) compared to responder T cells cultured without IL-2 (0.66%) (Panel A&B). Furthermore, the addition of IL-2 to the culture of responder T cells with BiP can further increase the frequency of Foxp+CD25+ cells (4.23%) compared to responder T cells cultured with IL-2 alone (3.40%). Data from 3 donors showed similar trend as shown in Panel E. Responder T cells cultured with BiP had lower frequency of Foxp3+CD25+ without IL-2 compared to responder T cells cultured without BiP (0.44% versus 0.54%). However, the addition of IL-2 increased the frequency of Foxp3+CD25+ cells within responder T cells from 2.16% to 2.61%.

The increase of Foxp3+CD25+ cells in responder T cells cultured with BiP at Day 4 (Figure 5.13(A)) was in parallel with an increase of IL-10 secretion from responder T cells cultured with BiP (Figure 5.12(E)). Therefore, IL-10 concentrations were measured from responder T cells cultured with or without BiP in the presence or absence of IL-2. The cumulative data from 3 donors was demonstrated in Panel F. Responder T cells cultured

with or without BiP produced very low levels of IL-10. However, responder T cells cultured with BiP in the presence of IL-2 produced 20 fold higher IL-10 in the culture compared to responder T cells cultured without BiP in the presence of IL-2 (124 pg/ml versus 5 pg/ml).

5.3.6 Responder T cells maintained an increased IL-10 secretion profile when cultured with BiP after being stimulated with anti-CD3/CD28 beads

To confirm the effect of BiP on responder T cells after 4 days in the culture, responder T cells were re-stimulated with anti-CD3/CD28 beads and the proliferation of these cells and cytokine profile was examined after 3 days as shown in Figure 5.15. The mean proliferation of stimulated responder T cells (BiP treatment) showed an increase in proliferation compared to stimulated responder T cells cultured (mock treatment) (17896 CPM versus 15867 CPM) but the difference was not statistically significant ($p=0.139$, $n=10$) as shown in panel A. The similar trend can be observed with all cytokine measured IFN-γ (Panel B), TNF-α (Panel C) and IL-10 (Panel D). IFN-γ and TNF-α secretions from stimulated responder T cells (BiP treatment) were higher compared to mock-treated responder T cells (IFN-γ: 688 pg/ml versus 361 pg/ml, TNF-α: 243 pg/ml versus 226 pg/ml) but the difference was not statistically significant (IFN-γ: $p=0.14$, $n=8$, TNF-α: $p=0.3$, $n=10$). However, the increase in IL-10 secretion in BiP-treated responder T cells (220 pg/ml) compared to mock-treated responder T cells (180 pg/ml) after re-stimulation with anti-CD3/CD28 beads is statistically significant ($p=0.0098$, $n=10$).

5.3.7 BiP-induced Foxp3+CD25+ cells can downregulate TNF-α in the co-culture

The investigation of the effect of BiP on responder T cells demonstrated that BiP can induce Foxp3+CD25+ cells and IL-10 secretion. These findings further support the contention that BiP may convert responder T cells into regulatory T cells. *In vitro* suppression assay is the gold standard used to determine the suppressive capacity of induced regulatory T cells. Hence, BiP treated responder T cells were co-cultured with autologous responder T cells to determine the ability of BiP-induced Foxp3+CD25+ to suppress responder T cells. Figure 5.16 illustrates the *in vitro* suppression of BiP-treated responder T cells (4 day treatment) compared with responder T cells cultured without BiP. Co-culture of BiP-treated responder T cells with autologous responder T cells reduced the proliferation in the co-culture compared to mock-treated responder T cells as shown in Panel A (39560 CPM v 42759 CPM). Similarly, IFN-γ, TNF-α and IL-10 secretions from the co-

culture of BiP-treated responder with autologous responder T cells were lower compared to mock-treated responder T cells (IFN-γ: Panel C, 5827 pg/ml versus 7773 pg/ml) (TNF-α: Panel E: 292 pg/ml versus 373 pg/ml) (Panel G: IL-10, 362 pg/ml versus 595 pg/ml).

The cumulative data followed the same trend as the individual donor. The proliferation (Panel B), IFN-γ (Panel D) and IL-10 (Panel H) data from multiple donors indicates the variability of response although the mean proliferation, IFN-γ and IL-10 from multiple donors from the co-culture of BiP-treated responder T cells with autologous responder T cells were lower than mock-treated responder T cells (Proliferation: 16552 CPM versus 17580 CPM, IFN-γ: 2306 pg/ml versus 2766 pg/ml, IL-10: 336 pg/ml versus 350 pg/ml). The variability in the response among donors made the reduction of proliferation, IFN-γ and IL-10 secretions induced by BiP treatment in responder T cells were not statistically significant (Proliferation: p=0.36, IFN-γ: p=1, IL-10: p=0.58, n=11 for all except IFN-γ, n=9). However, a different trend was observed with TNF-α secretion from the co-culture. TNF-α levels were reduced in 10 of 11 donors in the co-culture of BiP-treated responder T cells with autologous responder T cells and the mean TNF-α from BiP-treated responder T cells was signifcantly lower compared to mock-treated responder T cells as shown in panel F (295 pg/ml versus 355 pg/ml, p=0.024, n=11).

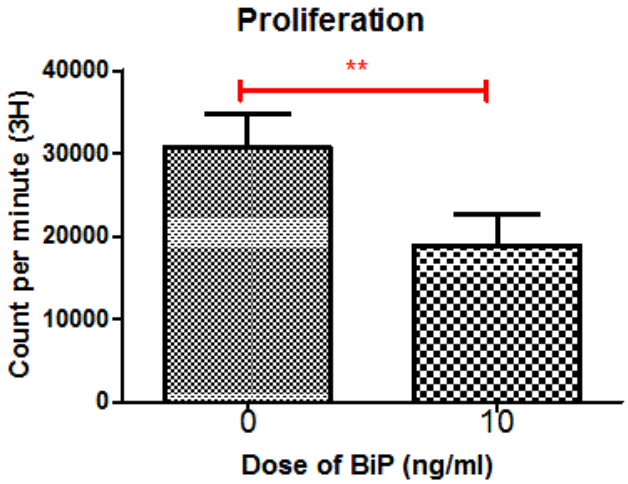


Figure 5.1 BiP can reduce responder T cell proliferation in the presence of anti-CD3/CD28 beads. This experiment was performed on cone bloods and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 4 days in the presence of 1 anti-CD3/CD28 bead to 5 cells with or without 10 ng/ml of BiP. Responder T cells were harvested after 4 days and proliferation was measured by tritiated thymidine incorporation for 16-18 hours. Each bar represents a mean of 8 donors±standard error of mean. Responder T cell proliferation was compared in the absence or presence of BiP using the Wilcoxon signed rank test. ** indicates p value <0.01.

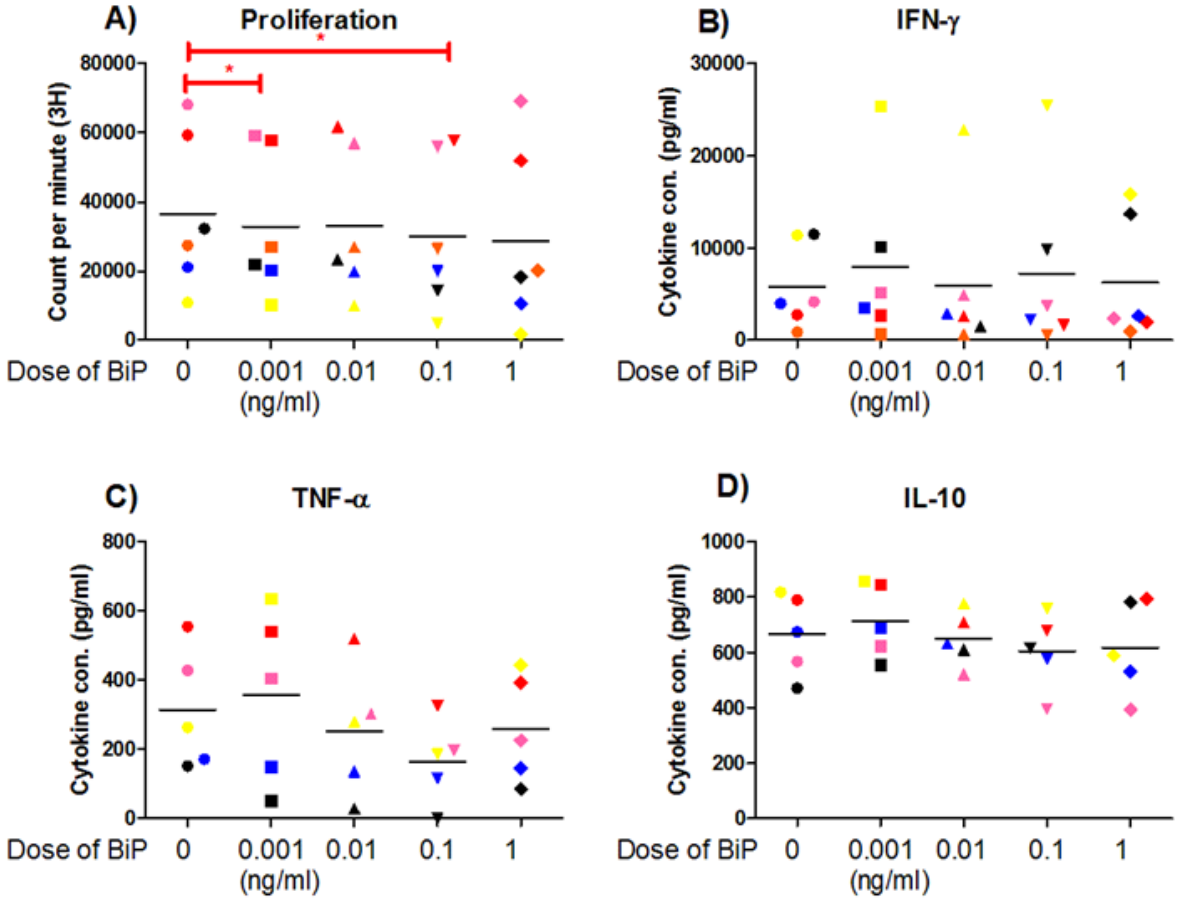


Figure 5.2 BiP can reduce proliferation of responder T cells in a dose-dependent manner. This experiment was performed on fresh bloods and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 4 days in the presence of 1 anti-CD3/CD28 bead to 5 cells with various doses of BiP (0.001, 0.01, 0.1 & 1 ng/ml). Responder T cells were harvested after 4 days and proliferation was measured by tritiated thymidine incorporation for 16-18 hours while culture supernatants were harvested after 4 days and cytokines were measured by ELISA. Panel (A) shows the proliferation of responder T cells while panel (B-D) demonstrate cytokine concentrations measured from the culture. Each dot represents a mean of 5 replicates of one donor for proliferation (A) while each dot in (B-D) represents a mean of triplicates of one donor and a matching colour indicates a similar donor. The Wilcoxon signed rank test was used to compare between responder T cells cultured with or without BiP and * indicates that p value is <0.05.

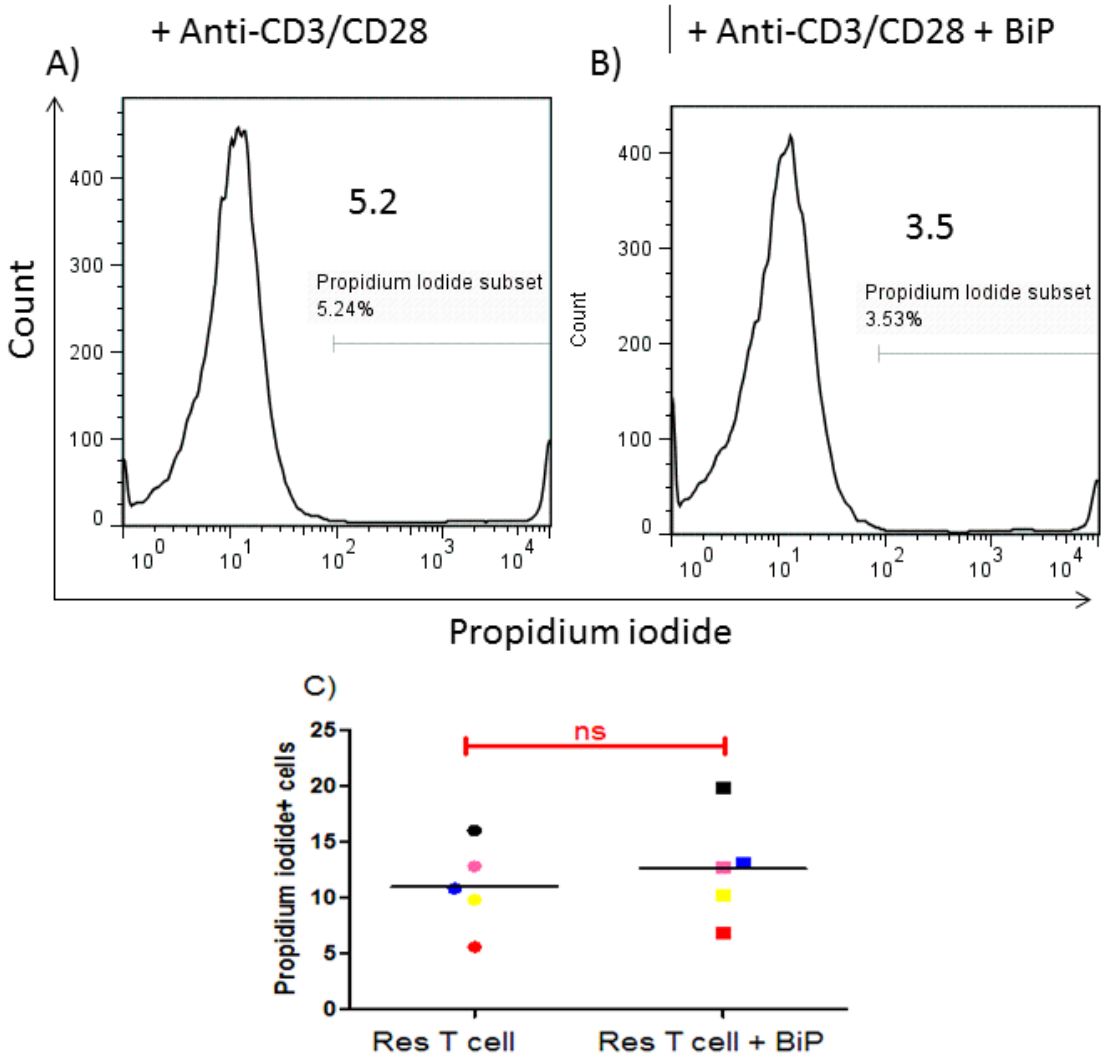


Figure 5.3 Reduction of responder T cell proliferation cultured with BiP in the presence of anti-CD3/CD28 beads is not due to an increase in dead cell. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 4 days in the presence of 1 anti-CD3/CD28 beads to 5 cells. Responder T cells were cultured in the absence or presence of 10 ng/ml BiP. Cells were stained with propidium iodide to measure cell death in the culture. Data was acquired using FACS Calibur and analysed by FlowJo software. Panel (A) shows a histogram of propidium iodide of responder T cells cultured without BiP while (B) shows responder T cells cultured with BiP. Each dot in (C) represents one measurement from 5 donors and a matching colour indicates a same donor. The Wilcoxon signed rank test was used to compare responder T cells cultured in the absence or presence of BiP and ns indicates that p value is >0.05.

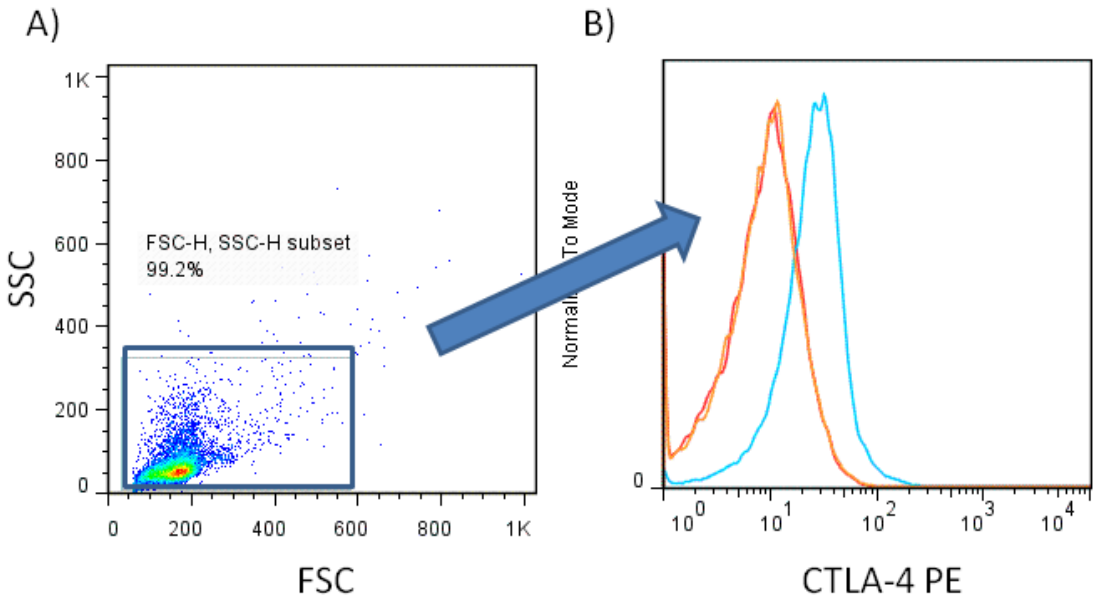
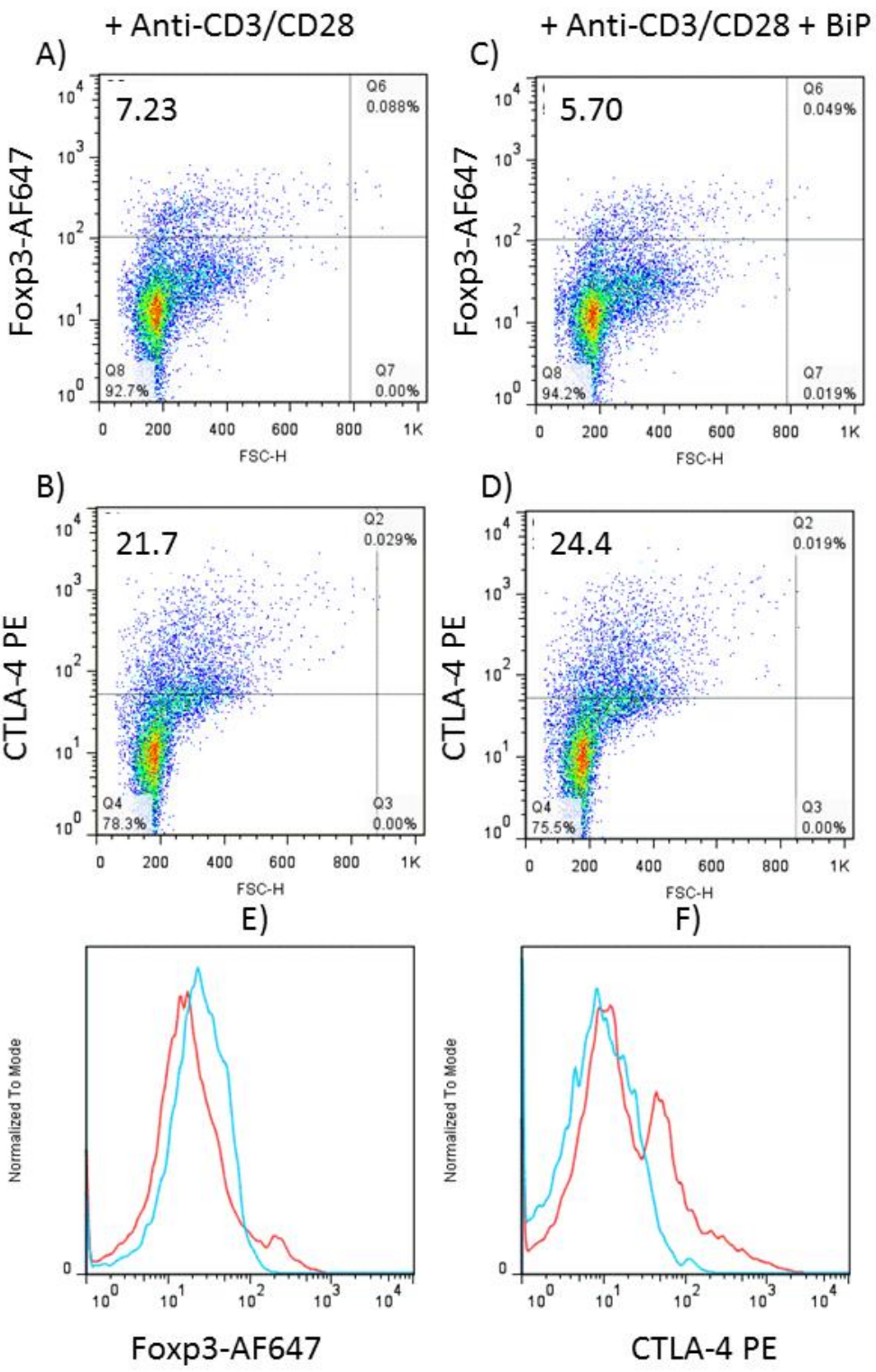


Figure 5.4 Fluorescence Minus One (FMO) was used to control for anti-CTLA-4 antibody staining instead of isotype control. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Intracellular staining of anti-CTLA-4 antibody was performed after cells were permeabilised using an intracellular staining kit from BioLegend. Panel (A) demonstrates Forward versus Side scatter of responder T cells after isolation while panel (B) shows a histogram of CTLA-4 comparing unstained (red line), isotype control (blue) and CTLA-4 antibody staining (orange). This figure is a representative of 3 donors.


+ Anti-CD3/CD28
+ Anti-CD3/CD28 + BiP
A)
C)
B)
D)
E)
F)
7.23
Q6
0.088%
Q8
92.7%
Q7
0.00%
5.70
Q6
0.049%
Q8
94.2%
Q7
0.019%
21.7
Q2
0.029%
Q4
78.3%
Q3
0.00%
24.4
Q2
0.019%
Q4
75.5%
Q3
0.00%
Foxp3-AF647
CTLA-4 PE
FSC-H
Normalized To Mode
Foxp3-AF647
CTLA-4 PE

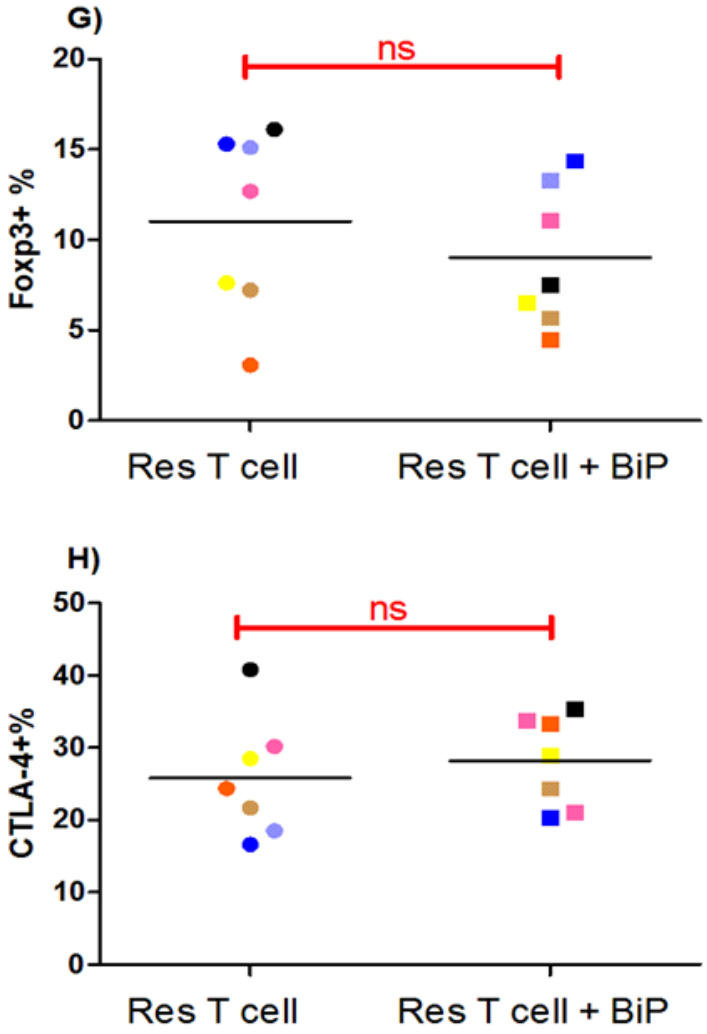


Figure 5.5. BiP does not induce any changes in Foxp3 or CTLA-4 expression within stimulated responder T cells. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 4 days in the presence of 1 anti-CD3/CD28 bead to 5 cells with or without 10 ng/ml of BiP. Responder T cells were harvested after 4 days and intracellular staining of Foxp3 and CTLA-4 were performed. Data was acquired using FACS Calibur and analysed by FlowJo software. Panel (A-B) show Foxp3 and CTLA-4 expression of stimulated responder T cells cultured in without BiP while panel (C-D) show responder T cells with BiP. Panel (E-F) shows a histogram of Foxp3 (E) and CTLA-4 (F) comparing antibody staining (red line) and control (blue line). Each dot in (G-H) represents one replicate of 7 donors and a matching colour indicates a same donor. The Wilcoxon signed rank test was used to compare responder T cells cultured with or without BiP and ns indicates that p value is >0.05.

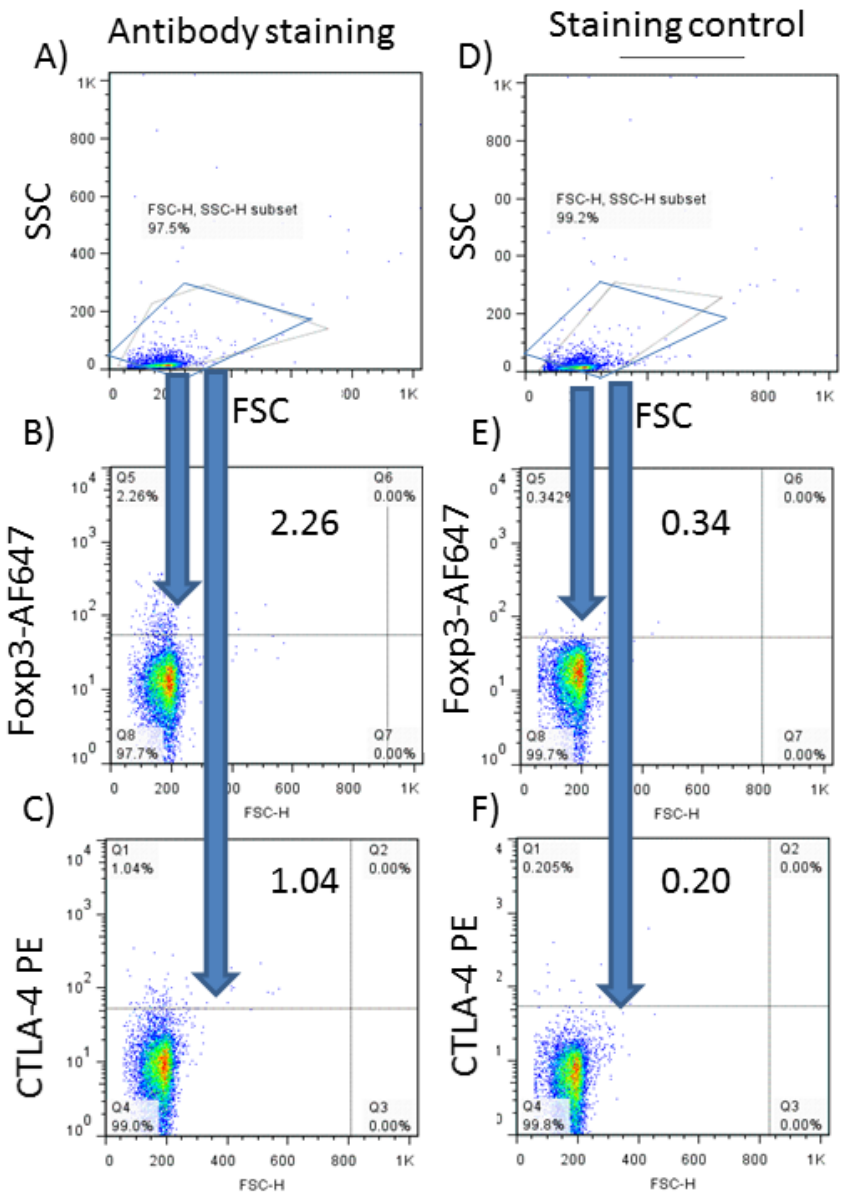
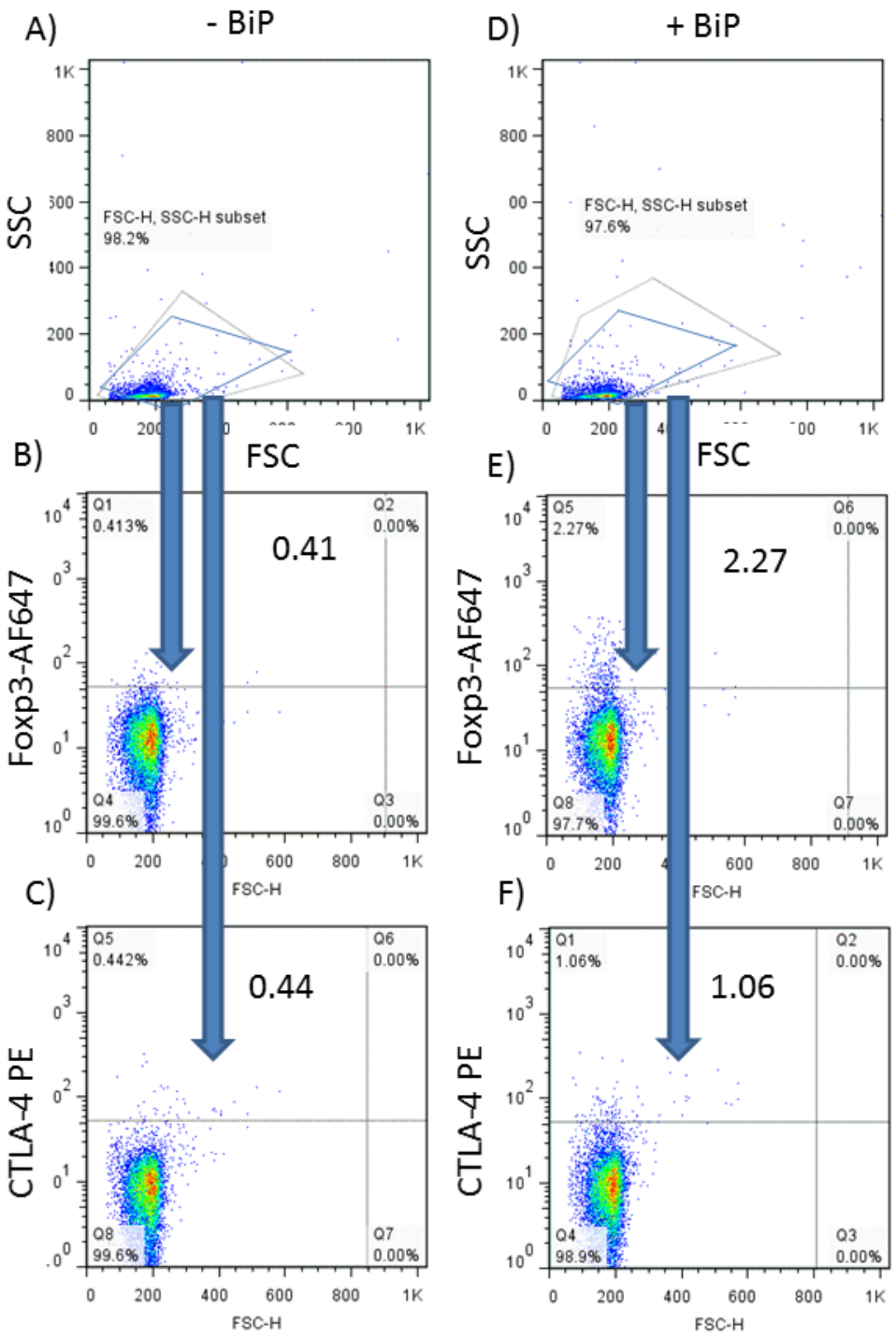


Figure 5.6 Anti-Foxp3 antibody staining was compared against isotype control while anti-CTLA-4 antibody was compared against FMO. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 4 days. Cells were harvested after 4 days and intracellular staining of Foxp3 and CTLA-4 was performed. Data was acquired using FACS Calibur and analysed by FlowJo software. Panel (A&D) show Forward versus Side scatter; Panel (B&D) show Foxp3 expression while panel (C&F) illustrate CTLA-4 expression of unstimulated responder T cells cultured with BiP comparing antibody staining (A-C) and staining control (D-F).


A)
- BiP
D)
+ BiP
SSC
FSC-H, SSC-H subset
98.2%
FSC-H, SSC-H subset
97.6%
FSC
B)
E)
Foxp3-AF647
Q1
0.413%
Q2
0.00%
0.41
Q4
99.6%
Q3
0.00%
Q5
2.27%
Q6
0.00%
2.27
Q8
97.7%
Q7
0.00%
FSC-H
C)
F)
CTLA-4 PE
Q5
0.442%
Q6
0.00%
0.44
Q8
99.6%
Q7
0.00%
Q1
1.06%
Q2
0.00%
1.06
Q4
98.9%
Q3
0.00%

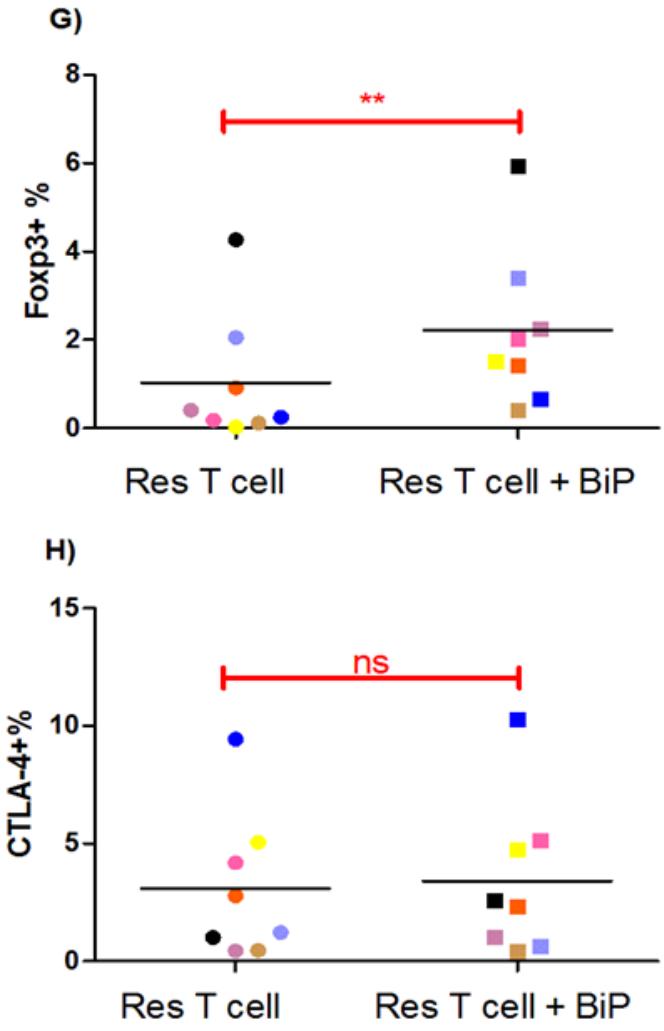
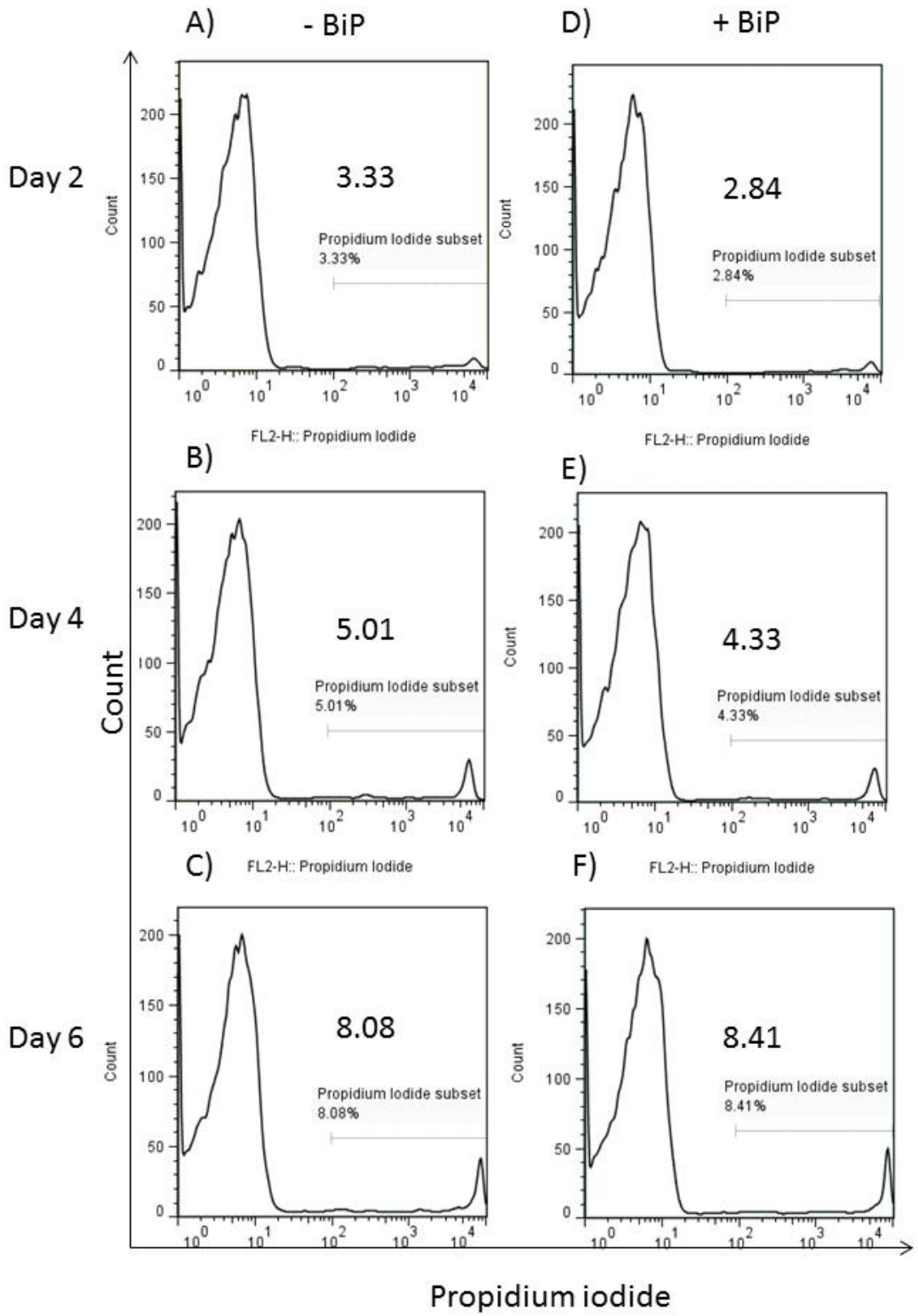


Figure 5.7. BiP induces an increase in Foxp3+ cells; but not CTLA-4+ cells within unstimulated responder T cells. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 4 days with or without 10 ng/ml of BiP. Responder T cells were harvested after 4 days and intracellular staining of Foxp3 and CTLA-4 was performed. Data was acquired using FACS Calibur and analysed by FlowJo software. Panel (A&D) show Forward versus Side scatter; panel (B&D) show Foxp3 expression while panel (C&F) illustrate CTLA-4 expression of unstimulated responder T cells comparing culture without (A-C) and with BiP (D-F). Panel (G-H) show the frequency of Foxp3+ (G) and CTLA-4+ (H) cells in multiple donors. Each dot in (G-H) represents one replicate of 8 donors and a matching colour indicates a same donor. The Wilcoxon signed rank test was used to compare responder T cells cultured with or without BiP. ** indicates that p value is <0.01 while ns indicates that p value is >0.05.


A)
- BiP
D)
+ BiP
Day 2
3.33
Propidium Iodide subset
3.33%
2.84
Propidium Iodide subset
2.84%
FL2-H:: Propidium Iodide
B)
E)
Day 4
5.01
Propidium Iodide subset
5.01%
4.33
Propidium Iodide subset
4.33%
Count
C)
F)
Day 6
8.08
Propidium Iodide subset
8.08%
8.41
Propidium Iodide subset
8.41%
Propidium iodide

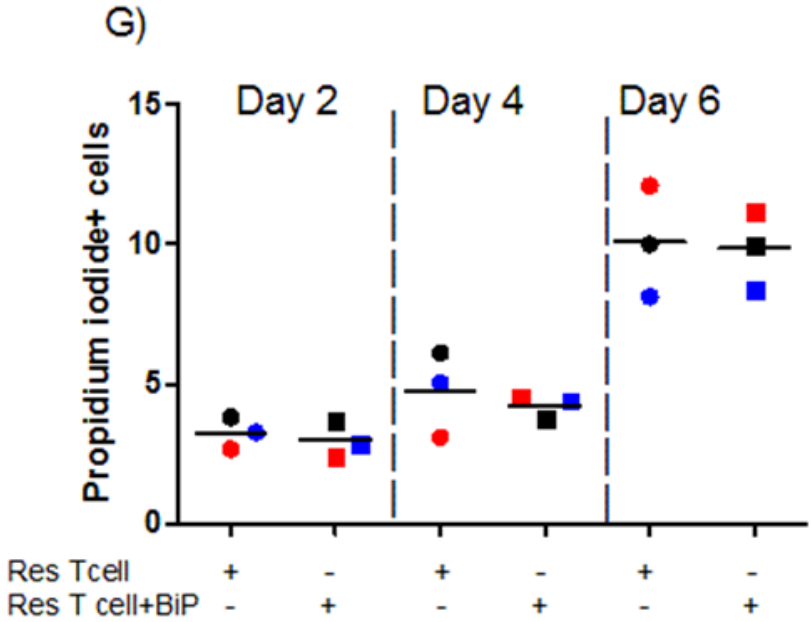


Figure 5.8. Increased cell death after 6 days of responder T cell in culture. This experiment was performed on fresh bloods and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with or without 10 ng/ml BiP for 2,4 and 6 days and cells were harvested and stained with propidium iodide. Data was acquired using FACS Calibur and analysed with FlowJo. Panel (A-C) show a histogram illustrating propidium iodide of responder T cells cultured without BiP while panel (D-F) show responder T cells cultured with BiP. Each dot in (G) represents one donor and a matching colour indicates a same donor.

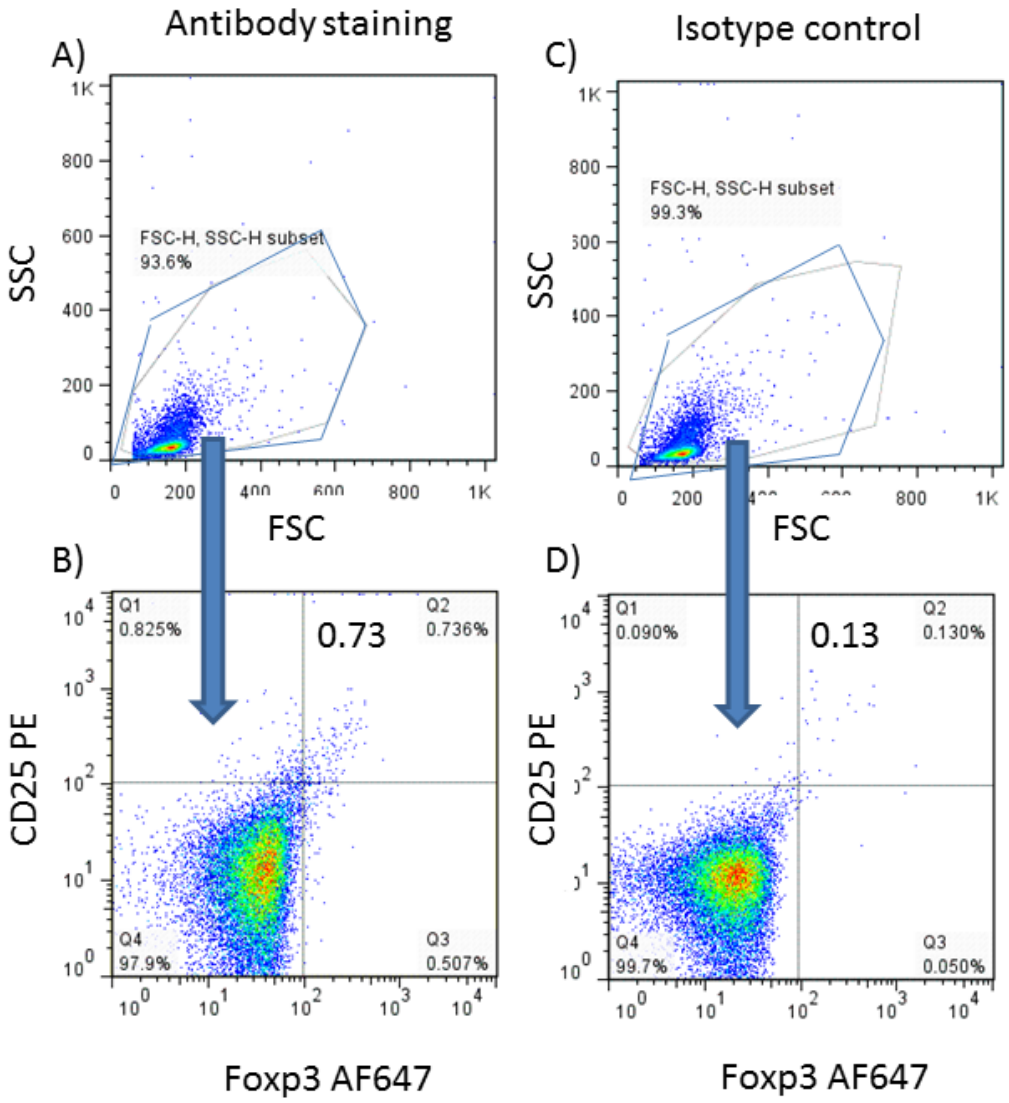
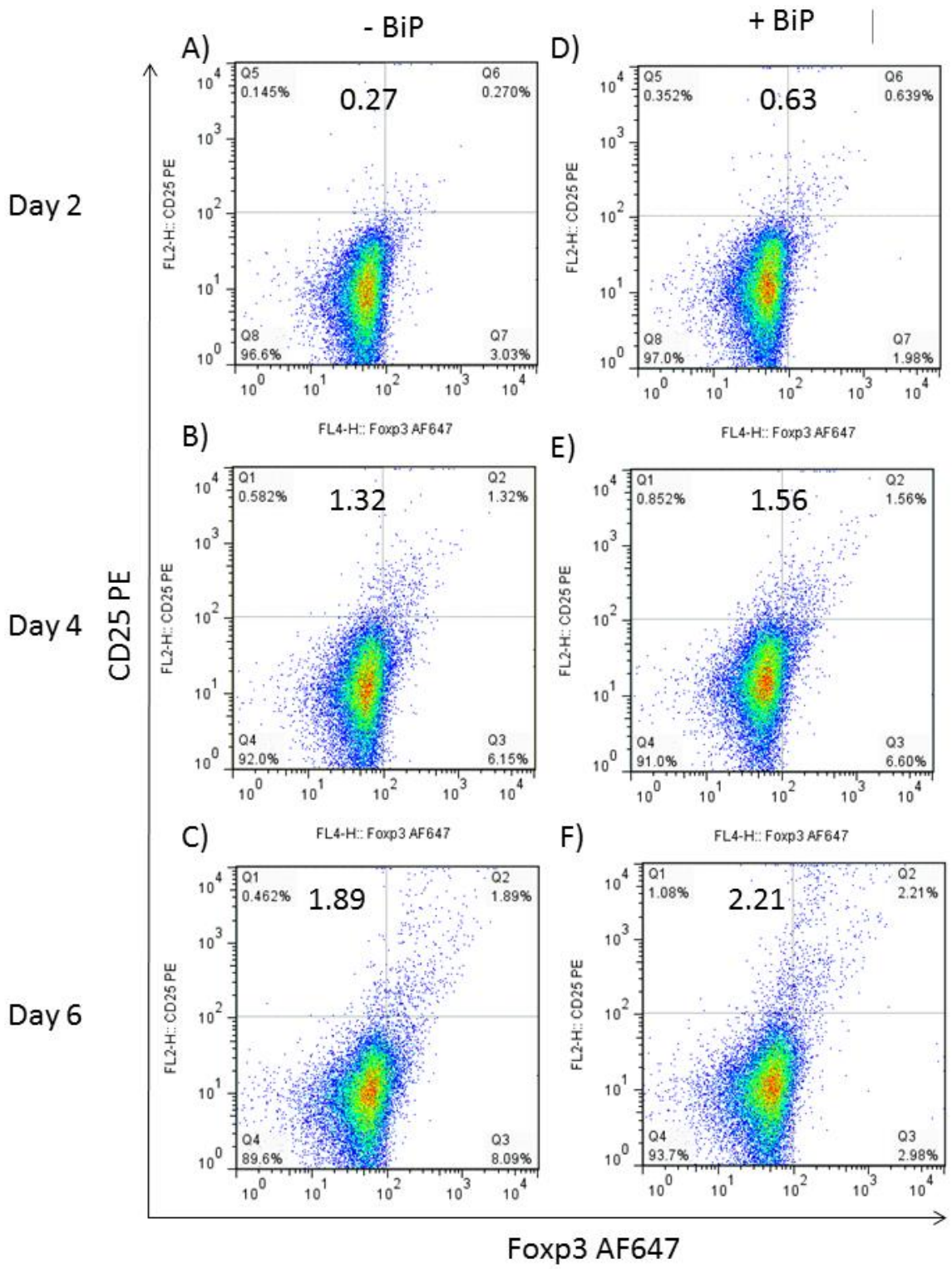


Figure 5.9 Anti-Foxp3 and anti-CD25 antibodies staining were compared against isotype control. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 4 days. Cells were harvested after 4 days and intracellular staining of Foxp3 and CTLA-4 was performed. Data was acquired using FACS Calibur and analysed by FlowJo software. Panel (A&C) show Forward versus Side scatter; Panel (B&D) CD25 versus Foxp3 expression of unstimulated responder T cells cultured without BiP comparing antibody staining (A-B) and isotype control (C-D).


- BiP
+ BiP
A)
D)
B)
E)
C)
F)
Day 2
Day 4
Day 6
CD25 PE
Foxp3 AF647
FL2-H:: CD25 PE
FL4-H:: Foxp3 AF647
0.27
0.63
1.32
1.56
1.89
2.21
Q5 0.145%
Q6 0.270%
Q8 96.6%
Q7 3.03%
Q5 0.352%
Q6 0.639%
Q8 97.0%
Q7 1.98%
Q1 0.582%
Q2 1.32%
Q4 92.0%
Q3 6.15%
Q1 0.852%
Q2 1.56%
Q4 91.0%
Q3 6.60%
Q1 0.462%
Q2 1.89%
Q4 89.6%
Q3 8.09%
Q1 1.08%
Q2 2.21%
Q4 93.7%
Q3 2.98%

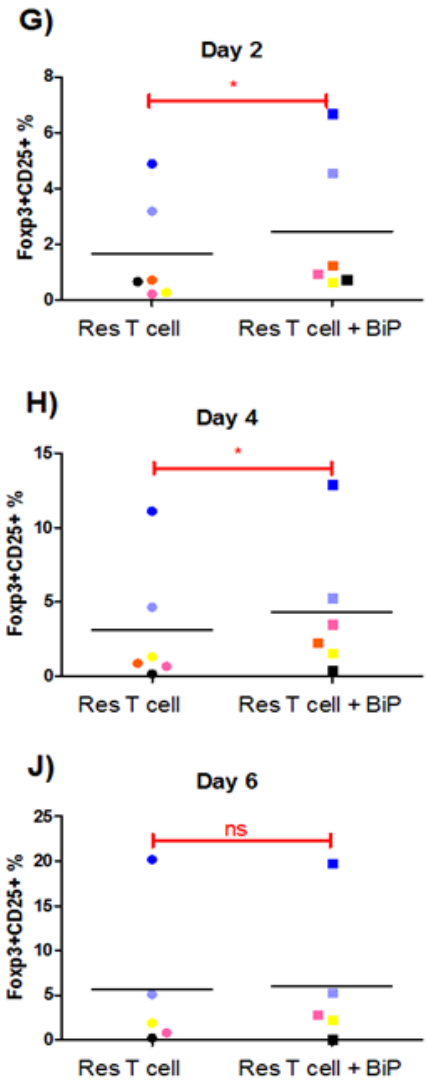


Figure 5.10 BiP induces an increase in the frequency of Foxp3+CD25+ cells on Days 2 and 4 within unstimulated responder T cells. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 2, 4 and 6 days with or without 10 ng/ml of BiP. Responder T cells were harvested and surface staining of CD25 and intracellular staining of Foxp3 were performed. Data was acquired using FACS Calibur and analysed by FlowJo software. Panel (A-C) show CD25 versus Foxp3 expression of responder T cells cultured without BiP while panel (D-F) illustrate responder T cells cultured with BiP. Panel (G-J) show the frequency of Foxp3+CD25+ cells on Day 2 (G), Day 4 (H) and Day 6 (J). Each dot in (G-H) represents one replicate of 6 donors and a matching colour indicates a same donor. The Wilcoxon signed rank test was used to compare responder T cells cultured with or without BiP. * indicates that p value is <0.05 while ns indicates that p value is >0.05.

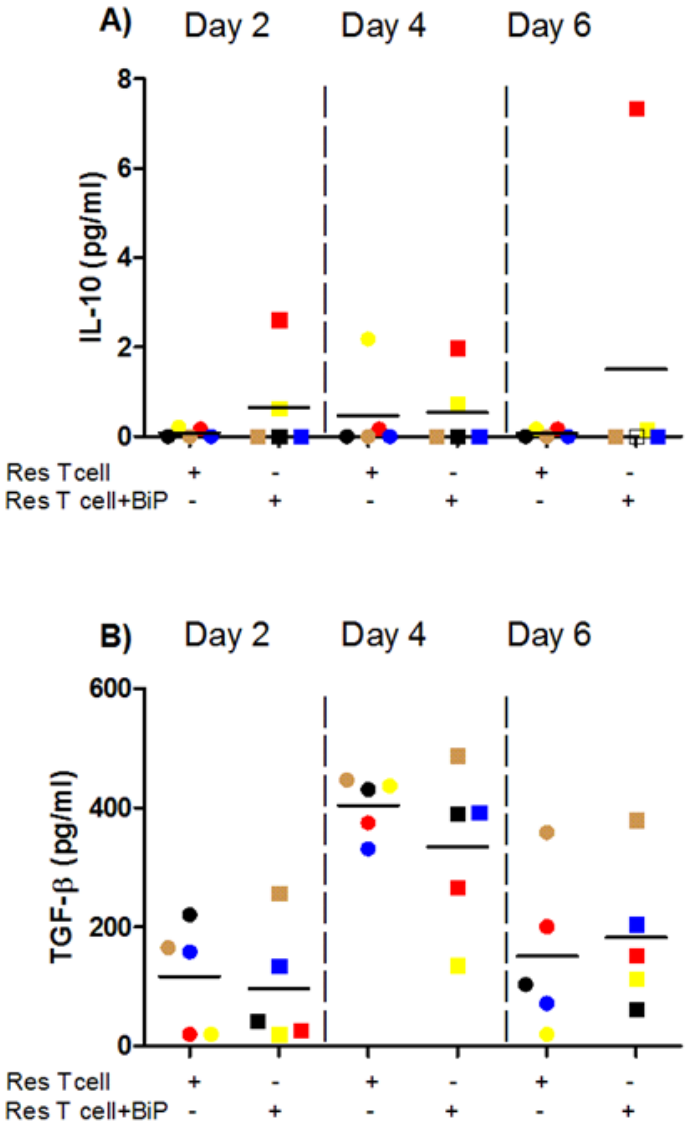


Figure 5.11 IL-10 levels were undetectable in most donors but BiP can reduce TGF-β secretion from responder T cell culture supernatants. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured with RPMI media supplemented with 10% FCS for 2, 4 and 6 days with or without 10 ng/ml of BiP. Culture supernatant were harvested at Days 2, 4 and 6 and IL-10 and TGF-β concentrations were measured using ELISA. Panel (A) shows IL-10 concentrations while panel (B) shows TGF-β concentrations from supernatant of responder T cell culture. Each dot in (A-B) represents a mean of triplicate measurements of one donor and a matching colour indicates a same donor.

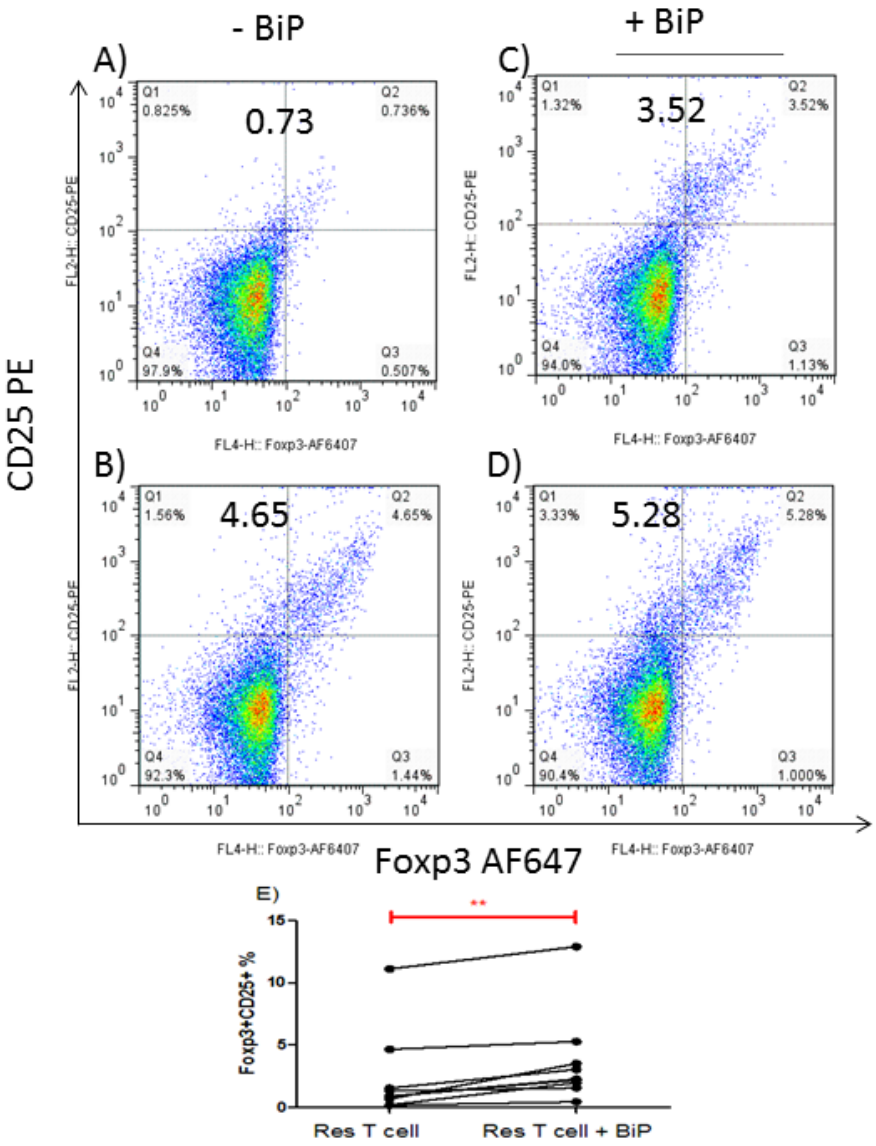


Figure 5.12. BiP can induce an increase in the frequency of Foxp3+CD25+ cells within unstimulated responder T cells. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured in RPMI media supplemented with 10% FCS for 4 days with or without 10 ng/ml of BiP. Responder T cells were harvested after 4 days and intracellular staining of Foxp3 and surface staining of CD25 was performed as described in Section. Data was acquired using FACS Calibur and analysed by FlowJo software. Panel (A-B) show CD25 versus Foxp3 expression of responder T cells cultured without BiP while panel (C-D) illustrate responder T cells cultured with BiP. Panel (A-B) demonstrate CD25 versus Foxp3 expression of one donor while panel (C-D) from another donor. Each dot in (E) represents one replicate of 9 donors. The Wilcoxon signed rank test was used to compare between responder T cells cultured with or without BiP and ** indicates that p value is <0.01.

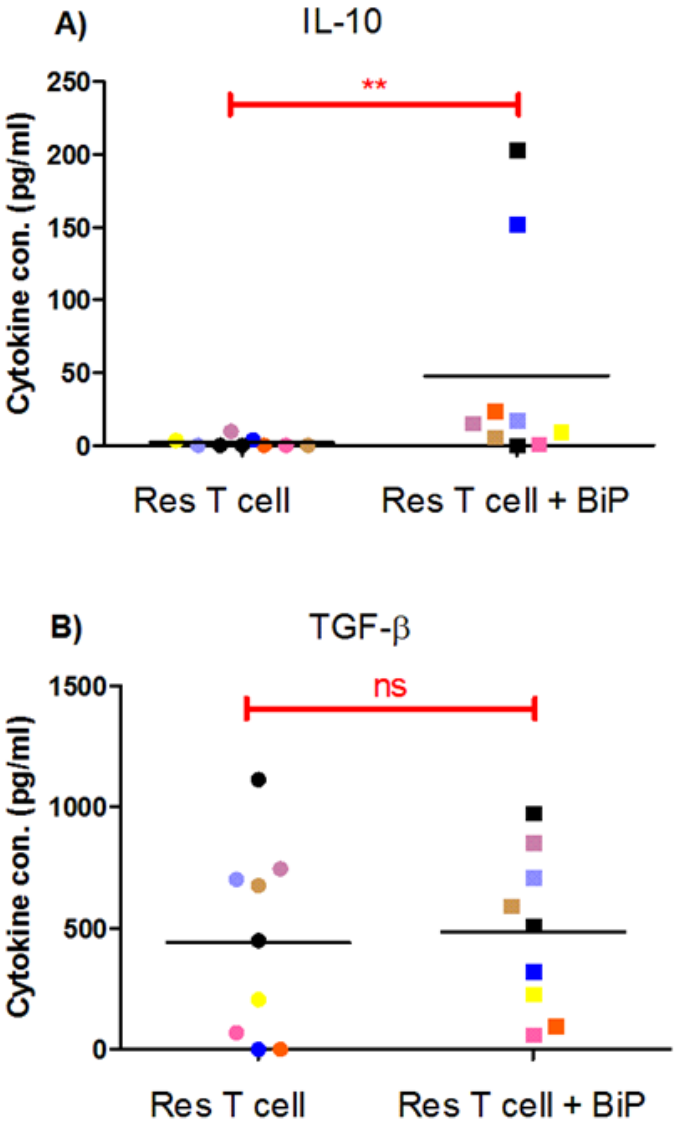
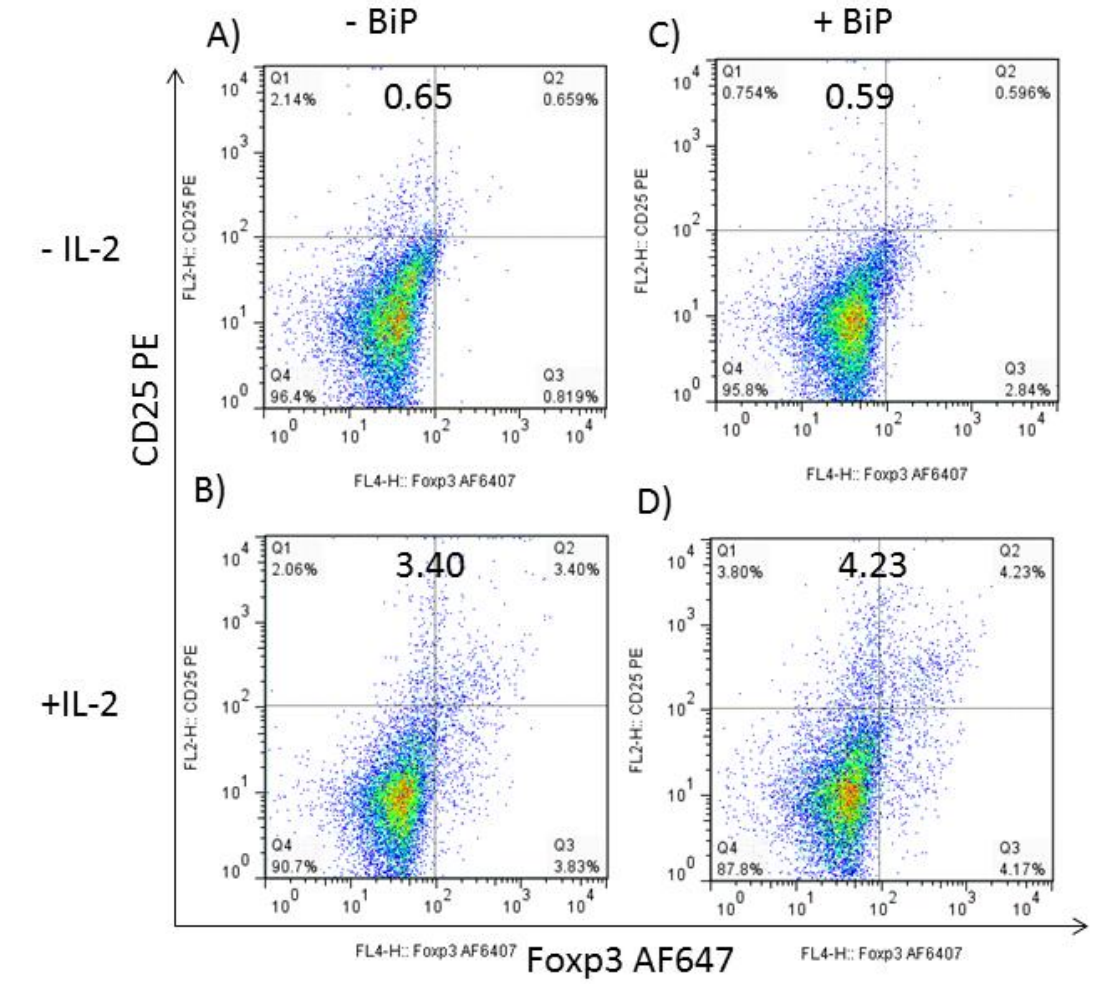


Figure 5.13. BiP can induce an increase in IL-10 secretion within unstimulated responder T cells. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured in RPMI media supplemented with 10% FCS for 4 days with or without 10 ng/ml of BiP. Culture supernatants were harvested after 4 days and IL-10 and TGF-β concentrations were measured using ELISA. Each dot in the figure represents a mean of triplicate measurements and a matching colour indicates a same donor. Panel (A) represents IL-10 concentrations in the culture of from multiple donors while panel (B) shows TGF-β concentration. The Wilcoxon signed rank test was used to compare responder T cells cultured with or without BiP and ** indicates that p value is <0.01.


A)
- BiP
C)
+ BiP
- IL-2
+IL-2
CD25 PE
B)
D)
0.65
Q1 2.14%
Q2 0.659%
Q4 96.4%
Q3 0.819%
0.59
Q1 0.754%
Q2 0.596%
Q4 95.8%
Q3 2.84%
3.40
Q1 2.06%
Q2 3.40%
Q4 90.7%
Q3 3.83%
4.23
Q1 3.80%
Q2 4.23%
Q4 87.8%
Q3 4.17%
FL2-H:: CD25 PE
FL4-H:: Foxp3 AF6407
Foxp3 AF647

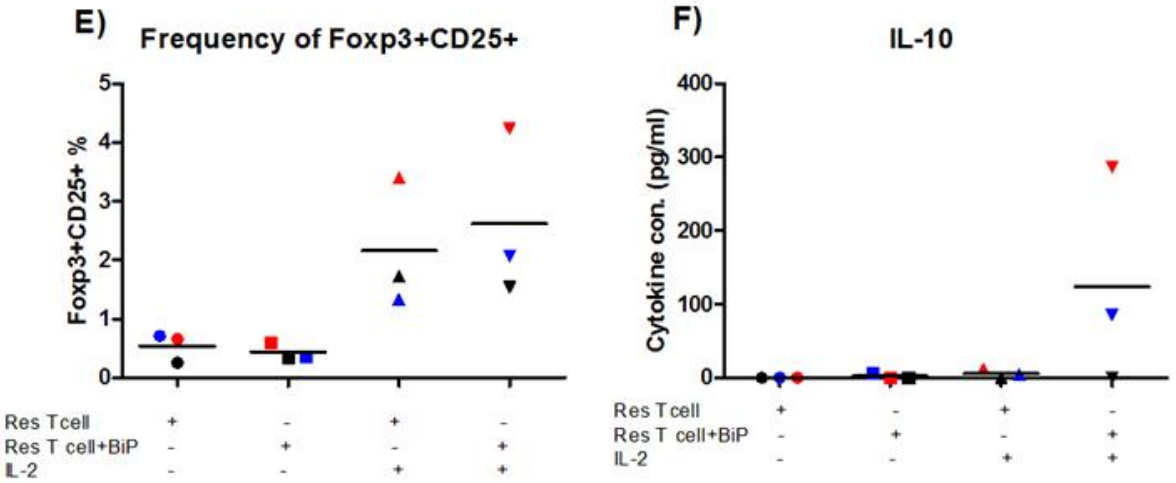

E)
Frequency of Foxp3+CD25+
Foxp3+CD25+ %
Res Tcell
Res T cell+BiP
IL-2
F)
IL-10
Cytokine con. (pg/ml)

Figure 5.14. IL-2 can induce an increase to the frequency of Foxp3+CD25+ but the combination of IL-2 and BiP can increase Foxp3+CD25+ frequency and IL-10 secretion within unstimulated responder T cells. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured in RPMI media supplemented with 10% FCS for 6 days with or without 10 ng/ml of BiP. Culture supernatants were harvested after 6 days and IL-10 and TGF-β concentrations were measured using ELISA. intracellular staining of Foxp3 and surface staining of CD25 was performed. Data was acquired using FACS Calibur and analysed by FlowJo software. Panel (A-B) show CD25 versus Foxp3 expression of responder T cells cultured without BiP while panel (C-D) illustrate responder T cells cultured with BiP. Panel (A&C) show CD25 versus Foxp3 expression of responder T cells cultured in the absence of IL-2 while (B&D) show responder T cells cultured in the presence of IL-2. Panel (E) shows the frequency of Foxp3+CD25+ while (F) shows IL-10 concentration measured from the culture supernatants. Each dot in (F) represents a mean of triplicate measurements of one donor and a matching colour indicates a same donor.

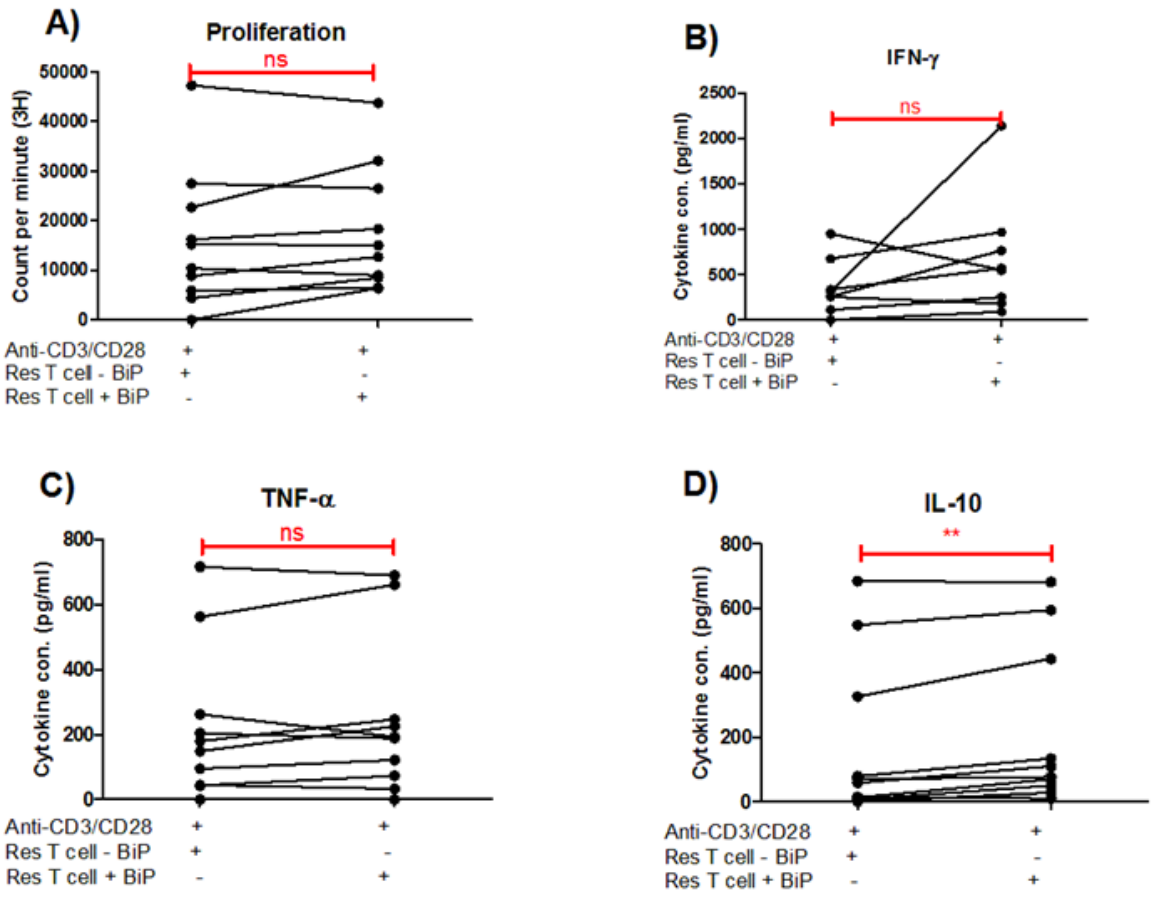
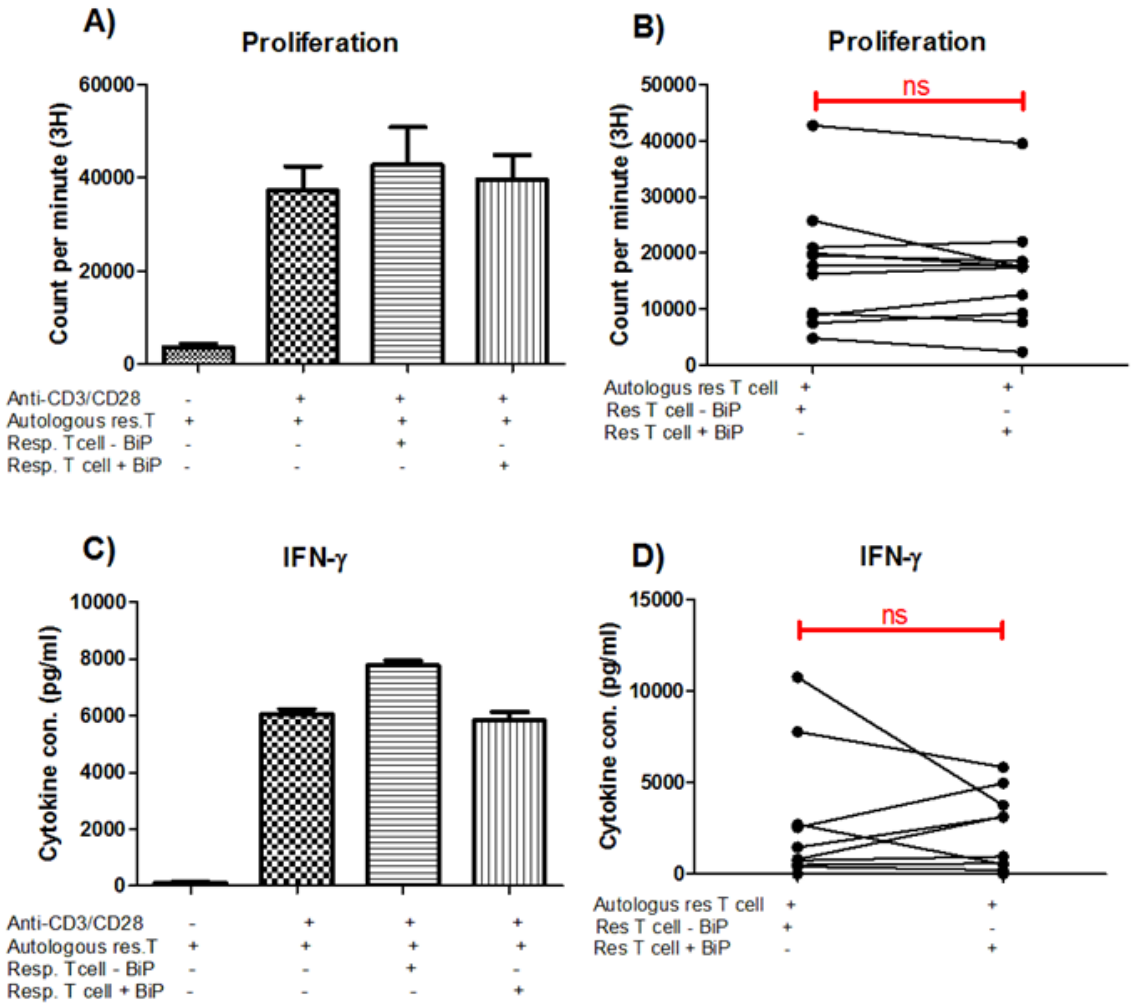


Figure 5.15. Re-stimulation of responder T cells after 4 days in culture with BiP increase IL-10 secretion. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured in RPMI media supplemented with 10% FCS for 4 days with or without 10 ng/ml of BiP. Responder T cells were harvested, recounted and re-suspended in new media before being cultured for 3 days in the presence of 1 anti-CD3/CD28 bead to 5 cells. Cell proliferation was measured by tritiated thymidine incorporation for 16-18 hours and culture supernatants were harvested after 4 days and cytokines were measured by ELISA. Panel (A) shows proliferation of responder T cells while panel (B-D) show cytokine concentrations measured from culture supernatants. Each dot represented in (A) is a mean of 5 replicates for one donor while (B-D) is a mean of triplicate measurements of one donor of 8 donors. The Wilcoxon signed rank test was used to compare responder T cells cultured with or without BiP. Ns indicates that p value is >0.05 while ** indicates that p value is <0.01.


A)
Proliferation
Count per minute (3H)
60000
40000
20000
0
Anti-CD3/CD28 - + + +
Autologous res.T + + + +
Resp. Tcell - BiP - - + -
Resp. T cell + BiP - - - +
B)
Proliferation
ns
Count per minute (3H)
50000
40000
30000
20000
10000
0
Autologus res T cell + +
Res T cell - BiP + -
Res T cell + BiP - +
C)
IFN-γ
Cytokine con. (pg/ml)
10000
8000
6000
4000
2000
0
Anti-CD3/CD28 - + + +
Autologous res.T + + + +
Resp. Tcell - BiP - - + -
Resp. T cell + BiP - - - +
D)
IFN-γ
ns
Cytokine con. (pg/ml)
15000
10000
5000
0
Autologus res T cell + +
Res T cell - BiP + -
Res T cell + BiP - +

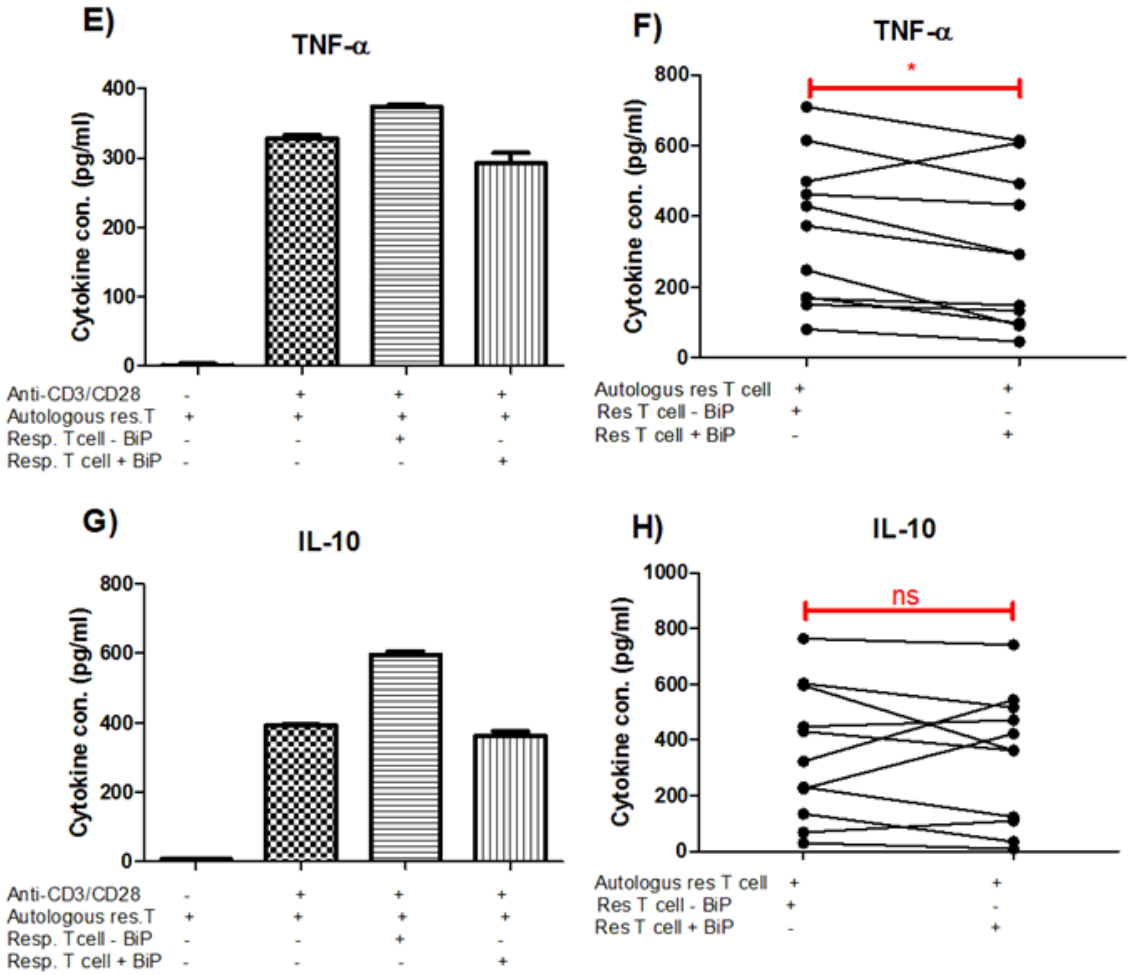


Figure 5.16. BiP-induced Foxp3+CD25+ cells reduce TNF-α secretion from autologous responder T cell in co-culture. This experiment was performed on fresh blood and responder T cell isolation was performed as shown in Figure 4.1. Responder T cells were cultured in RPMI media supplemented with 10% FCS for 4 days with or without 10 ng/ml of BiP. Responder T cells were harvested, recounted and re-suspended in new media before being cultured for 3 days in the presence of 1 anti-CD3/CD28 beads to 5 cells and co-cultured with autologous responder T cells. Cell proliferation was measured by tritiated thymidine incorporation for 16-18 hours. Panel (A) shows cell proliferation and each bar represents a mean of 5 replicates while panel (B) shows cell proliferation in multiple donor as each dot represents one donor. Panel (B,D,F) show cytokine concentration measured from culture supernatants and each bar represents a mean of triplicate measurements while panel (C,E,G) illustrate cytokine concentration in multiple donors as each dot represents one donor. The Wilcoxon signed rank test was used to compare autologous responder T cells co-cultured with responder T cells treated with or without BiP. NS indicates that p value is >0.05 while * indicates that p value is <0.05.

5.4 Discussion

This chapter was based on the finding from the previous chapter as shown in Figure 4.18(B) where responder T cells stimulated with anti-CD3/CD28 beads in the presence of BiP can reduce their proliferation compared to stimulated responder T cells alone. The titration of BiP showed that the proliferation of responder T cells stimulated with anti-CD3/CD28 beads demonstrated a dose-dependent reduction of proliferation as show in Figure 5.2(A). However, the variability of response from multiple donors in cytokine secretion profile (IFN-γ, TNF-α and IL-10, Figure 5.2(B-D)) made the difference between the mean of cytokine secretions of 0 ng/ml and various doses (1, 0.1, 0.01 and 0.001 ng/ml) of BiP statistically insignificant. These findings may indicate that BiP convert a small percentage of responder T cells to become regulatory T cells as Corrigall et al (2009) reported that BiP can induce regulatory T cell development via BiP-treated DC. Before further experiment was performed, viability staining was performed to determine if BiP may induce cell death in responder T cells. Data shown in Figure 5.3 demonstrate that BiP did not induce an increase of dead cells as indicated by propidium iodide staining further supporting the hypothesis that BiP may induce regulatory T cells. Vercoulen and colleagues (2009) showed that human regulatory T cells did not induce cell death in activated responder T cells.

Regulatory T cells induced by BiP-treated DC were characterised by the presence of CD25 and CD27 on their surface and increased intracellular CTLA-4 expression but not Foxp3 expression (Corrigall et al, 2009). Therefore a co-stain of intracellular CTLA-4 and Foxp3 was performed on responder T cells stimulated with anti-CD3/CD28 beads as shown in Figure 5.5. Clearly, the frequency of both CTLA-4+ and Foxp3+ cells were not modulated by BiP presence in the culture. However, as mentioned before, CTLA-4 and Foxp3 can be expressed by responder T cells after activation. Therefore, to see a direct effect of BiP on responder T cells, responder T cells were cultured in the absence of anti-CD3/CD28 beads with or without BiP as demonstrated in Figure 5.7. BiP presence in the culture significantly increased the frequency of Foxp3+ cells within responder T cells, but not CTLA-4+ cells. This observation suggest a novel effect of BiP on responder T cells and further strengthen the contention that BiP may induce regulatory T cell development.

To confirm this observation, a kinetic experiment of BiP effect was performed (shown in Figure 5.8-5.11). Human regulatory T cells can be identified by the presence of

CD25 and Foxp3 molecules; therefore a co-stain of these two antibodies were performed in the kinetic experiment. Figure 5.10 shows that BiP can induce an increase of Foxp3+CD25+ cells after 2 and 4 days in the culture but not after 6 days. In addition, percentages of dead cells in the culture increased two fold on Day 6 compared to Day 4. Therefore, responder T cell culture was repeated for 4 days and the data shown in Figure 5.12 with more donors (n=9 compared to n=5 in Figure 5.10) confirmed that BiP can induce an increase of Foxp3+CD25+ population within responder T cells. To support the contention that BiP can convert human CD4+CD25- cells into regulatory T cells, regulatory T cells-associated cytokines IL-10 and TGF-β were measured from the supernatants. IL-10 levels were only detected in one donor after BiP treatment in Figure 5.11(A) but data from Figure 5.13(A) indicated that responder T cells increased their IL-10 secretion when cultured with BiP. In contrast, TGF-β secretions from responder T cells cultured with BiP were approximately similar to responder T cells cultured without BiP (Figure 5.13(A)). To confirm BiP effect on responder T cells, responder T cells were cultured in the presence of IL-2 for 6 days as IL-2 is an important survival factor for regulatory T cells. Data from kinetic experiments (Figure 5.10(J)) suggest that BiP cannot increase Foxp3+CD25+ cells at Day 6, hence this time point was chosen. Figure 5.14 show that IL-2 can increase Foxp3+CD25+ cells within responder T cells but not with IL-10 secretion. However, the combination of BiP and IL-2 in the culture could act in synergy to increase Foxp3+CD25+ population and simultaneously increase IL-10 secretion indicating that BiP may act on responder T cells.

The function of BiP-induced Foxp3+CD25+ cells were examined to support the contention that BiP can induce regulatory T cell development. BiP-treated responder T cells were re-stimulated with anti-CD3/CD28 beads and their proliferation and cytokine profiles were investigated as illustrated in Figure 5.15. The most important observation from this figure is that responder T cells maintained enhanced IL-10 secretion even after re-stimulation with anti-CD3/CD28 beads in the absence of BiP. This observation recapitulated the previous work by shown by Dr. Corrigall's group. Bodman-Smith et al (2003) showed that BiP can induce IL-10 secretion in CD8+ T cells while Corrigall et al (2004) demonstrated that human PBMCs cultured with BiP can induce IL-10 secretion and human dendritic cells cultured with BiP can induce copious amounts of IL-10 as shown by Corrigall et al (2009).

Finally, it is essential to examine whether all these changes to responder T cells can lead to enhanced suppressive function in *in vitro* suppression assay. The data in Figure 5.16(B) demonstrated that 7 out of 11 donors of BiP-induced Foxp3+CD25+ cells can suppress the proliferation of autologous responder T cells but the difference is not statistically significant. As shown in Figure 5.12(E), BiP can induce approximately 3.7% Foxp3+CD25+ cells within responder T cells compared to 2.3% Foxp3+CD25+ cells within responder T cells cultured without BiP. Therefore, the final ratio of Foxp3+CD25+ cells in the co-culture of BiP-treated responder T cells with autologous responder T cells is approximately 1 to 50. Verma et al (2009) demonstrated that natural or thymus-induced regulatory T cells activated with a specific alloantigen can suppress naive CD4+ T cells in the ration of 1:32-64. Therefore, it is plausible that the suppressive capacity of BiP-induced Foxp3+CD25+ was masked by the high ratio of Foxp3+CD25+ cells to responder T cells (CD4+CD25-Foxp3-) in the co-culture.

Although no significant effect was observed on proliferation and other cytokine profile (IFN-γ and IL-10, Figure 5.16(D&H)), an effect can be observed in TNF-α levels measured from the co-culture. BiP-induced Foxp3+CD25+ cells can downregulate TNF-α secretion from non-treated responder T cells (Figure 5.16(F)). The downregulation of TNF-α from the co-culture by BiP-induced Foxp3+CD25+ cells may be related with enhanced IL-10 secretion. This relationship was observed by Corrigall et al (2004) in which they showed that BiP can induce an increase in IL-10 and reduction in TNF-α concentrations measured from PBMC culture. Reciprocal evidence further supports the contention that IL-10 can regulate TNF-α level. Evans and colleagues (2014) demonstrated that culturing human CD4+ T cells with various TNF-α inhibitors can induce an increase in the frequency of IL-10+ cells within CD4+ T cells. To further confirm this effect, utilising the neutralising antibody against IL-10 should be used in the co-culture to confirm that the downregulation of TNF-α from responder T cells is due to enhanced IL-10 secretion.

This chapter demonstrates that BiP can induce an increase in Foxp3+CD25+ cells and IL-10 secretion within responder T cells. This may lend support to the hypothesis that BiP can induce the development of regulatory T cells characterised by the presence of Foxp3 and CD25 molecules. In addition, BiP-induced Foxp3+CD25+ cells showed specificity in cytokine secretion by secreting more IL-10 instead of TGF-β. By comparison, regulatory T

cells cultured with BiP for 4 days showed an increased in Foxp3 protein expression but secreted more TGF-β instead of IL-10 as demonstrated in the previous chapter. However, BiP-induced Foxp3+CD25+ cells failed to suppress the proliferation of the autologous responder T cells in the co-culture. Tran and co-workers (2007) argued that Foxp3 induction in human CD4+CD25- in the presence of TCR stimulation and TGF-β does not confer a regulatory phenotype and thus failed to suppress the proliferation of responder T cells. In contrast, Zheng and colleagues (2004) demonstrated that human CD4+CD25- cells can be induced to become CD4+CD25+ cells with regulatory phenotype and these regulatory cells can suppress using cell-to-cell contact and TGF-β and IL-10. However, these two papers demonstrated that Foxp3 can be induced in CD4+CD25- cells in the presence of TCR stimulation and cytokines namely IL-2 or TGF-β.

The results demonstrated that BiP may induce Foxp3+CD25+ regulatory T cells; therefore it is appropriate to compare the result with HSP-induced regulatory T cells. de Kleer et al (2010) showed that HSP60, an example of HSP, can induce Foxp3+ regulatory T cells after 6 days of responder T cell culture with HSP60 in the presence of monocytes. In addition, Aalberse and colleagues (2011) demonstrated that HSP60-induced Foxp3+ T cells from human cord blood can suppress effector T cell response *in vitro*. Furthermore, van Herwijnen and co-workers (2012) demonstrated that Foxp3+ regulatory T cells can be induced by HSP70 (a member of HSP70 family) immunisation in mice. Therefore, it is possible that BiP can induce Foxp3+ regulatory T cell development as reported with other HSPs.

The inability of BiP-induced Foxp3+CD25+ cells to suppress responder T cells may due to technical problem. To overcome this problem, BiP-induced Foxp3+CD25+ cells must be sorted before being co-cultured with autologous responder T cells with lower ratio. However, another marker has to be used to identify BiP-induced Foxp3+CD25+ cells as Foxp3 is an intracellular protein which cannot be used as a marker to sort this population. de Kleer et al (2010) indicated that CD30 molecules present on the surface of HSP60-induced regulatory T cells could be used as an additional marker to sort HSP60-induced regulatory T cells before suppression assays were performed. In addition, van Herwijnen and co-workers (2012) demonstrated that HSP70-induced regulatory T cells in mice can be identified by the presence of Lymphocyte Activation Gene 3 (LAG3) molecules on their

surface, one of the surface molecules associated with the regulatory T cell mechanism of suppression. Therefore, it is crucial to identify another surface marker that can be used to sort BiP-induced Foxp3+CD25+ cells before these cells can be co-cultured with autologous responder T cells.

This chapter demonstrates BiP can induce Foxp3+CD25+ cells within responder T cells while the previous chapter shows that BiP may enhance Foxp3 expression in regulatory T cells. The similarity of BiP effects on responder and regulatory T cells can be seen on enhanced Foxp3 expression. As discussed in the previous chapter, BiP may bind to a receptor and mediate signalling via the STAT5 molecule as the downstream target of STAT5 is the Foxp3 protein. In addition to comparing the suppressive capacity of BiP-treated responder T cells and mock-treated responder T cells, Foxp3 localisation can be examined to determine whether BiP-induced Foxp3+CD25+ cells were truly regulatory T cells as Foxp3 protein measured using flow cytometry cannot determine the exact location of this protein. Magg et al (2012) reported that human CD4+CD25- T cells can express Foxp3 upon activation. However, they compared the localisation of Foxp3 protein between human CD4+CD25- after activation and CD4+CD25+ regulatory T cells and found that Foxp3 was localised in the cytoplasm in activated CD4+CD25- T cells while the Foxp3 protein in regulatory T cells was localised in the nucleus. The localisation of Foxp3 can help to reveal whether Fopx3 induction in BiP-treated responder T cells may convert responder T cells to regulatory T cells.

However, the obvious effect of BiP on responder T cells can be seen in the induction IL-10 secretion in the culture (Figure 5.13) and after re-stimulation with anti-CD3/CD28 beads in the absence of BiP (Figure 5.15). The signalling pathway involved in IL-10 secretion in responder T cells can be demonstrated in the previous studies. Corrigall et al (2004) and Corrigall et al (2009) both showed that IL-10 secretion by PBMC and dendritic cells can be partially blocked using neutralising antibodies towards p38 MAPK. P38 MAPK is composed of 4 subunits; α, β, γ and δ and can be activated by activated by a variety of environmental stresses and inflammatory cytokines. Ng and colleagues (2013) showed in their review showed that IL-10 expression can be induced by various STAT molecules including STAT1, 3, 4 and 6. Zhang et al (2004) showed that p38 MAPK is the upstream molecule involved in STAT1 phosphorylation and STAT1 phosphorylation was blocked in p38 MAPK mutant cells.

However, blocking p38 MAPK was effective in partial blockade of IL-10 secretion by BiP as shown by Corrigall et al (2004) and Corrigall et al (2009). Therefore, it is possible that IL-10 induction in responder T cells utilises another signalling pathway involving one of the STAT molecules. The most likely pathway utilised by BiP to induce IL-10 secretion is via STAT3 phosphorylation. Hossain and co-workers (2013) demonstrated that Foxp3 can act as a co-transcription factor with STAT3 for IL-10 transcription in tumour-induced regulatory T cells. In addition, regulatory T cells derived from breast cancer patients can secrete IL-10. Taking these results together, BiP may induce IL-10 secretion by signalling through STAT1 and STAT3.

IL-10 secretion in BiP-induced Foxp3+CD25+ cells may further add support to the contention that BiP can convert responder T cells to regulatory T cells by a new pathway. Firstly, BiP can indirectly convert responder T cells to CD4+CD25+CD27+ regulatory T cells via dendritic cells derived monocytes cultured with BiP (Corrigall et al, 2009). In addition, an increase of TGF-β secretion by regulatory T cells cultured with BiP may indirectly convert responder T cells to regulatory T cells in the presence of BiP as Zheng et al (2002) showed that TGF-β and a low-dose antigen can induce human CD4+CD25- to become Th3 suppressor cells, an induced regulatory T cell characterised by the presence of Foxp3, and they can secrete TGF-β. Lastly, BiP may directly induce regulatory T cell development with or without IL-2 and these cells are characterised by the presence of Foxp3 protein and can secrete IL-10 as demonstrated in this chapter.

5.5 Conclusion

The conclusions of this chapter are as follows:-

- BiP can reduce proliferation of responder T cells stimulated with anti-CD3/CD28 beads in a dose-dependent manner after 4 days in culture
- BiP can induce Foxp3+CD25+ cells within a responder T cell population and induce IL-10 secretion
- BiP-induced Foxp3+CD25+ cells can downregulate TNF-α secretion from autologous responder T cells in the co-culture

Chapter 6

General discussion

6 Discussion

6.1 Soluble BiP is biologically active and its activity is not due to LPS contamination

Soluble BiP may modulate regulatory T cells and can affect responder T cells in the previous chapters. Regulatory T cells cultured with BiP may enhance Foxp3 expression and induce TGF-β secretion. Furthermore, BiP can induce Foxp3+CD25+ cells within responder T cells and induce IL-10 secretion. These BiP-induced Foxp3+CD25+ cells may reduce TNF-α secretion from autologous responder T cells. However, this BiP effect was prominent in cytokine secretion in terms of IL-10 secretion compared to induction of Foxp3+CD25+ cells within responder T cells. BiP induced 30% more TGF-β in BiP-treated regulatory T cells compared to mock-treated regulatory T cells (Figure 4.31 (B)) while responder T cells cultured with BiP produced 25 times more IL-10 compared to responder T cells cultured without BiP (Figure 5.13 (A)). In contrast, regulatory T cells cultured with BiP showed a 11% increase in Foxp3 expression although the increase is statistically insignificant (p=0.06) as measured by MFI (Figure 4.30(I)) compared to regulatory T cells cultured without BiP whereas BiP induces 50% more Foxp3+CD25+ cells when responder T cells were cultured with BiP compared to responder T cells cultured without BiP. This observation leads to the question of whether other factors can further influence BiP activity.

Posttranslational modification to BiP may be essential for BiP to exert its immunoregulatory properties. One of the ways to detect the presence of posttranslational modification to protein to is search for autoantibodies towards modified BiP. In addition to detecting anti-BiP antibody in RA patients, Shoda et al (2011) reported the presence of anti-citrullinated BiP antibodies. Citrullination is a posttranslational modification to a protein where arginine residue is converted to non-coding amino acid citrulline. In the same study, higher levels of anti-citrullinated antibodies were detected in RA patients compared to anti-BiP antibody. Lu et al (2010) demonstrated the pathogenicity of anti-citrullinated antibodies by binding to citrullinated BiP on the surface of monocytes to stimulate TNF-α secretion. In addition, Shoda et al (2011) showed that immunisation of citrullinated BiP in mice exacerbated arthritis in the CIA model emphasising the pro-inflammatory role of citrullinated BiP in RA development. This is clearly in contrast to the observation made in

the experiment where BiP can induce immunoregulatory phenotypes in regulatory and responder T cells.

The BiP protein used in the experiment was a protein prepared using Good Manufacturing Practice (GMP) protocol. GMP BiP was produced to ensure no contamination of endotoxin in the protein preparation. Therefore, the BiP effect on regulatory and responder T cells was not due to any endotoxin including LPS contamination. One of the problems in HSP or stress protein research is endotoxin contamination, especially LPS. Lewkowicz and colleagues (2006) examined the effect of LPS on human regulatory T cells. Regulatory T cells cultured with 0.1 up to 1000 ng/ml of LPS demonstrated a reduction in Foxp3 expression as measured by Foxp3 MFI and this effect was not dose-dependent. This is in contrast with the BiP effect observed on regulatory T cells where BiP can increase Foxp3 expression when regulatory T cells were cultured with BiP.

To further confirm soluble BiP protein is biologically active, it is important to compare the BiP effect with unrelated proteins. The previous work done by our collaborator, Dr. Corrigall, demonstrated that the BiP effect is specific, and not simply due to protein structure. Previous works published by Corrigall et al (2001 and 2004) and Bodman-Smith et al (2003) compared the effect of BiP on various immune cells with β-galactosidase, an unrelated protein. The comparison was made because BiP and β-galactosidase were produced and raised in the similar organism, *Escherichia coli*. In addition, Brownlie (2004) demonstrated that FiTC-labeled BiP can bind to various immune cells including monocytes, B cells, and T cells compared to FiTC-labeled Human Serum Albumin (HSA). She showed that BiP can bind up to 58% of monocytes, 14% of B cells and 4.7% of T cells confirming that BiP binding to its receptor was specific compared to HSA.

6.2 Soluble BiP exerts its biological effect on regulatory and responder T cells by binding to a receptor

This observation brings us to the question of the identity of BiP receptor as Corrigall et al (2003) reported that the identity of the BiP receptor remains elusive. A literature search was performed to find any report of BiP receptors. Yang et al (2013) reported the interaction between BiP and other family members of HSP70 namely HSc70 and GRP75 and mannose receptor (MR), a member of C-type lectin receptor which is a pattern recognition

receptor. They showed MR binding with BiP in HeLa cells using co-immunoprecipitation technique. After searching the identity of the peptide sequence that binds to MR using proteomics and mass spectrometry, they confirmed the interaction between MR and BiP by Western blot analysis, ligand binding analysis and intracellular colocalisation using confocal microscopes. However, Geijtenbeek and Gringhuis (2009) reported that this receptor is only present in myeloid dendritic cells and macrophages and binds to high mannose, fucose and sulphated sugars. Based on this information, BiP certainly does not bind on MR as MR expression is limited to dendritic cells and macrophages and BiP is a protein whereas sugar is part of a carbohydrate. Therefore, it is highly unlikely BiP binds to MR to exert its effect on regulatory and responder T cells.

Recently the identity of the BiP receptor was revealed by our collaborator, Dr. Corrigall. BiP can bind to epidermal growth factor receptor (EGFR) before it can induce downstream signalling to mediate any effect. However, since this information is bound to intellectual property, we can only speculate whether EGFR can act as a BiP receptor by linking the effect of BiP on regulatory and responder T cells (cytokine secretion and Foxp3 expression) with the possible transcription factor involved in mediating these effects. However, the distribution of EGFR expression on regulatory and responder T cells was first examined based on the current literature. As shown in Figure 4.24 (A), 2 hours of BiP pre-treatment on monocytes can reduce the proliferation of responder T cells while it took 4 days for BiP to exert its effect on regulatory T cells demonstrated by a small increase in Foxp3 expression (Figure 4.30 (I)) and TGF-β secretion (Figure 4.31) and responder T cells as illustrated by an increase in the frequency of Foxp3+CD25+ cells (Figure 5.12) and enhanced IL-10 secretion (Figure 5.13). Given the time difference taken by BiP on monocytes and regulatory and responder T cells, it is certainly related to the distribution of BiP receptors as earlier report (Corrigall et al (2003)) indicated that the BiP receptor is highly expressed in monocytes compared to T cells. Chan and co-workers (2009) demonstrated that EGFR protein is highly expressed on human CD14+ monocytes as it can be detected using flow cytometry compared to its very low expression in CD3+ T cells. However, Zaiss et al (2013) reported that the RNA of EGFR can be detected on both human regulatory and responder T cells compared to protein level as demonstrated by Chan et al (2009). Interestingly, EGFR RNA is expressed 5 times more in human regulatory T cells compared to responder T cells

and the expression of the receptor was increased in murine regulatory T under inflammatory conditions. This supports the contention that this BiP receptor, namely EGFR, is highly expressed on monocytes compared to regulatory or responder T cells.

To further confirm that EGFR is a BiP receptor, the downstream signalling of EGFR was examined based on current literature. Then, the possible downstream signalling pathway utilised by EGFR was compared with our putative transcription factor involved in mediating the BiP effect as stated in the previous paragraph. The common effect of BiP on regulatory and responder T cells is inducing Foxp3 expression. As discussed in previous chapters, STAT5 is an important transcription factor involved in Foxp3 induction. Nakamura et al (1996) showed that EGFR signalling can induce phosphorylation in STAT5 and transduce a growth signal in haematopoietic cells. In addition, Beier and co-workers (2012) used EGFR expression as readout of STAT5 signalling in murine regulatory T cells. However, BiP induces a specific effect on regulatory and responder T cells in term of cytokine secretions. Regulatory T cells cultured with BiP may induce higher TGF-β while higher IL-10 secretion was induced in responder T cells cultured with BiP compared to responder T cells cultured without BiP. We speculated that BiP may induce secretion of TGF-β by signalling via p38 MAPK kinase and GLI2 whereas signalling via STAT1 and STAT3 may be involved in the BiP induction of IL-10 within responder T cells. Haura and colleagues (2005) reported that EGFR signalling in breast cancer tumour can induce the activation of STAT1, STAT3 and STAT5. However, a report by Haura et al (2005) did not explain how TGF-β signalling may be initiated by GLI2 transcription.

Based on this evidence, it is postulated that BiP binds to EGFR receptors to exert its effect on both regulatory and responder T cells. Ladanyi and Pao (2008) showed in their review how EGFR receptor signalling can activate STAT1 and STAT3 which may induce IL-10 while STAT5 activation by EGFR may induce Foxp3 expression and p38 MAPK activation for TGF-β induction. This hypothetical signalling of BiP binding to EGFR is illustrated in Figure 6.1 (regulatory T cells) and 6.2 (responder T cells). To confirm that BiP receptor is EGFR, anti-EGFR antibody can be used to block BiP binding to EGFR in regulatory and responder T cells. However, the big question remains is how BiP signalling can yield different outcomes in regulatory and responder T cells in term of cytokine secretion.

6.3 Soluble BiP concentrations are reduced in RA patients and influenced by ageing; therefore it may not induce immunoregulatory phenotype in regulatory and responder T cells in RA patients

Corrigall et al (2001) and Blass et al (2001) independently demonstrated the role of BiP as an autoantigen in RA. This raises an important question based on our observation on the effect of BiP on regulatory and responder T cells. In the previous two chapters, it was shown that BiP may modulate regulatory T cell phenotype and can affect responder T cell phenotype and function from healthy donors. One of the reasons why inflammation persists in RA patients is related to soluble BiP concentration. Although Blass et al (2001) showed that BiP was overexpressed in synovial section of RA patient compared to osteoarthritis (OA) patient, Shields et al (2011) reported that soluble BiP concentrations were significantly reduced in the circulation compared to OA patients. Lower concentration of soluble BiP could partly explained why inflammation persists in RA as BiP effect on regulatory and responder T cells and previous works (Corrigall et al, 2001, Bodman-Smith et al, 2003, Corrigall et al, 2004, Corrigall et al, 2009) demonstrated the immunoregulatory properties of BiP on various immune cells.

Another factor that may be important in the induction and secretion of BiP is age. The BiP effect on regulatory and responder T cells were obtained from cone bloods (age is unknown) and fresh bloods from young donors (20-35 years old). Therefore, it is plausible that soluble BiP induction and secretion is impaired in ageing hence the soluble BiP concentration produced is insufficient to induce immunoregulatory properties in regulatory and responder T cells as RA is more common in the elderly population. In response to acute sleep deprivation, Naidoo and colleagues (2008) reported that aged mice do not display an increase of BiP expression as UPR, the main inducer of BiP, can induce higher BiP expression in young mice after acute sleep deprivation. Furthermore, they showed that the basal expression of BiP decreased in aged mice compared to young mice. To confirm that aging can influence soluble BiP concentration in healthy donors, correlation analysis was performed between age and soluble BiP concentration from the Whitehall II cohort. Data from 68 donors did not demonstrate significant correlation between age and soluble BiP concentration. However, data from a larger cohort (n=189) demonstrated a significant correlation. Soluble BiP negatively correlates with age even though the correlation is a weak

correlation as indicated by the low correlation coefficient (R=-0.2027, p=0.0051). Correlation between age and soluble BiP concentration is demonstrated in Figure 6.3. This data explains that soluble BiP concentrations are reduced as the healthy donors from this cohort get older.

6.4 RA microenvironment is important to influence regulatory and responder T cell phenotype and function in RA patients

Synovial joints of RA patients are infiltrated with various immune cells and cytokines. One of the most important cytokines present in the joints is TNF-α. TNF-α has been shown to play various roles in the pathogenesis of RA as shown by Brennan and McInnes (2008). Not only is TNF-α responsible for inducing pro-inflammatory immune responses, TNF-α also plays a role in angiogenesis, endothelial cell activation, osteoclast activation and hepcidin induction and all of these process play an important role in RA pathogenesis. In addition, anti-TNF-α blockade has been licensed for use in RA patients. This underlines the importance of TNF-α in modulating the phenotype and function of immune cells especially regulatory and responder T cells.

TNF-α has been shown to impair the ability of regulatory T cells to suppress the proliferation of responder T cells or cytokines secreted by responder T cells. Valencia and colleagues (2006) demonstrated that regulatory T cells from RA patients had reduced mRNA Foxp3 expression and failed to suppress the proliferation of responder T cells, and this effect is reversed upon anti-TNF-α therapy. In addition, *in vitro* co-culture of human regulatory and responder T cells with TNF-α impaired the ability of regulatory T cells to suppress the proliferation of responder T cells as shown by Valencia et al (2006) and van Amelsfort et al (2007). In addition, Ehrenstein and colleagues (2004) showed that regulatory T cells from RA patients failed to suppress TNF-α and IFN-γ secretions from responder T cells in co-culture with regulatory T cells before anti-TNF-α treatment. However, the regulatory T cell capacity to inhibit pro-inflammatory cytokines secreted from responder T cells was restored upon anti-TNF-α therapy. Furthermore, Ehrenstein et al (2004) and Huang et al (2012) reported an increase in regulatory T cell frequency after anti-TNF-α treatment compared to regulatory T cell frequency before the treatment.

The ability of TNF-α to modulate regulatory T cell function is dependent on its ability to bind on TNF receptor II (TNFRII) on regulatory T cells as reported by Valencia et al (2006). This receptor is constitutively expressed on unstimulated regulatory T cells and the expression of this receptor can be upregulated by TNF-α. Nie et al (2013) performed an elegant experiment demonstrating the molecular mechanism of TNF-α in inhibiting human regulatory T cell function. They showed that phosphorylation of Ser418 in the C-terminus DNA binding domain in Foxp3 gene is important for human regulatory T cell suppressive function. TNF-α has been shown to impair the suppressive function of regulatory T cells in RA patients by inducing the protein-phosphatase 1 (PP1) enzyme. This enzyme can dephosphorylate Ser418 on the Foxp3 gene thus impairing the suppressive function of regulatory T cells. This effect was confirmed in RA patients receiving anti-TNF-α blockade treatment in which regulatory T cell suppressive function was restored which was associated with decreased expression of PP1 and increased Foxp3 phosphorylation.

Another possible mechanism of TNF-α inhibition on regulatory T cell function is by influencing the formation of immunological synapse (IS) between regulatory T cells and APCs. TNF-α inhibition of regulatory T cell function can be achieved by interrupting two signalling molecules in regulatory T cells, namely the protein kinase C- theta (PKC-θ) and the disc large homolog 1 (Dlgh1). PKC-θ is a major isoform of the protein kinase C that has been shown in to be involved in CD3-CD28 costimulation while Dlgh1 is a scaffold protein that may be involved in the regulation of T cell migration and receptor signalling. Zanin-Zhorov et al (2010) reported that PKC-θ sequestration in regulatory T cells during IS is necessary and the inhibition of PKC-θ in regulatory T cells was defective in RA patients. On the other hand, Zanin-Zhorov et al (2012) showed the opposite role of Dlgh1 during IS formation in regulatory T cells. Dlgh1 recruitment is necessary to mediate the suppressive activity of regulatory T cells and also was defective in regulatory T cells isolated from RA patients. Interestingly, defective PKC-θ sequestration and Dlgh1 recruitment can be reversed in regulatory T cells of RA patients by TNF-α inactivation.

In addition to modulating regulatory T cell function, regulatory T cell phenotypes can be affected by the microenvironment in the joints of RA patients. Walter and colleagues (2013) demonstrated that co-culture of human regulatory T cells and *in vitro* activated monocytes which mimics the monocytes present in the joints of RA patients can induce

regulatory T cells to secrete more cytokines including TNF-α, IFN-γ, IL-17 and IL-10 even though these regulatory T cells still maintain their suppressive capacity. In addition, Wang and co-workers (2014) showed that IL-17 producing regulatory T cells were present in RA patients with normal suppressive function. They also demonstrated that Th17 differentiation *in vitro* can be induced by the presence of IL-6 and IL-23 cytokines in *in vitro* culture. Gomez-Puerta et al (2013) reported that IL-6 concentration in the synovial fluid of RA patients was the highest concentration of cytokines measured compared to 16 other cytokines. In addition, the anti-IL-6 receptor antibody is now being licensed for use in clinics to treat RA.

Several studies have reported the effect of tocilizumab, an anti-IL-6 receptor antibody towards correcting the balance between regulatory T cells and Th17 cells. Samson and colleagues (2012) reported that tocilizumab treatment on RA patients can significantly decrease Disease Activity Score in 28 joints (DAS 28), Th17 cell frequency and increase percentages of regulatory T cells in 15 patients. In contrast, Pesce et al (2013) only reported an increase in regulatory T cell frequency; but no change was observed in Th17 cell frequency. By using adalimumab or anti-TNF-α antibody, regulatory T cells from RA patients can restrain or suppress Th17 cells by inhibiting monocyte-derived IL-6 as shown by McGovern et al (2012). They demonstrated that anti-TNF-α antibody administration to RA patients can induce an increase in Foxp3+ regulatory T cells that lacks Helios expression. In addition, IL-6 blockade in RA patients has been shown to expand CD39+ regulatory T cell frequency as reported by Thiolat et al (2014). Further work by Herrath and colleagues (2014) showed that CD39+ regulatory T cells isolated from rheumatic joints had the ability to suppress pro-inflammatory cytokines from responder T cells namely IFN-γ, TNF-α and IL-17A. Clearly, TNF-α and IL-6 play a significant role in modulating regulatory T cell phenotype and function in RA patients.

In addition, these two cytokines also have been shown to affect responder T cells by modulating their function. TNF-α and IL-6 can partially induce hyperactivation of effector T cells from RA patients hence rendering them resistant to suppression by regulatory T cells as reported by Wehrens et al (2011). The increased resistance of effector T cells to suppression by regulatory T cells was because of enhanced activation of protein kinase B (PKB)/c-akt in effector T cells and Haufe and colleagues (2011) reported an increased

resistance to suppression of synovial CD4+CD25- T cells from juvenile idiopathic arthritis indicating that this phenomenon maybe common in autoinflammatory conditions. The effect of TNF-α modulation on effector or CD4+CD25- T cells was not only limited to affecting their function but can also influence their frequency. Anti-TNF-α therapy can induce decreased frequency of activated CD4+ T cells but not regulatory T cells as reported by Dombrecht et al (2006). Furthermore, anti-TNF-α treatment can induce a specialised population of regulatory T cells in RA patients from responder T cells as shown by Nadkarni et al (2007). These induced CD4+CD25+Foxp3+ regulatory T cells were characterised by the absence of CD62 Ligand (CD62L) molecules on their surface and can mediate suppression using TGF-β and IL-10. In conclusion, regulatory and responder T cell phenotype and function can be modulated in an RA microenvironment especially by TNF-α and IL-6 as demonstrated in Figure 6.4.

6.5 How may soluble BiP restore defective regulatory and responder T cell function in RA patients?

As shown in the previous two chapters, soluble BiP may affect regulatory T cell phenotype but can influence responder T cell phenotype and function. Responder T cells cultured with BiP can induce an increase in Foxp3+CD25+ cells after 4 days. BiP-induced Foxp3+CD25+ cells can induce IL-10 secretion and these cells can downregulate TNF-α secretion from autologous responder T cells. The data showed that these cells may be induced regulatory T cells however the suppressive effect of these cells were variable as demonstrated in Chapter 5. To ensure that these cells are suppressive, additional surface markers need to be used to identify these cells before these cells can be sorted and co-cultured with autologous responder T cells. Nevertheless, these cells demonstrated the ability to modulate TNF-α from autologous responder T cells. As demonstrated by anti-TNF-α therapy, this clearly indicates that BiP may help in reducing the TNF-α level in RA microenvironment which may consequently lead to the resolution of inflammation. Furthermore, the reduction of TNF-α level may help to restore the defective function of regulatory T cells as demonstrated in the previous section.

In addition, regulatory T cells cultured with BiP may increase Foxp3 expression and TGF-β secretion. TGF-β is a pleiotropic cytokine which can affect various immune cells but the main focus is on DC. Andersson and colleagues (2008) showed that the role of

CD4+Foxp3+ regulatory T cells in maintaining tolerance in mice is dependent on TGF-β by conferring infectious tolerance. Further work by Lan et al (2012) showed that tolerogenic DC can be induced by TGF-β secreted by polyclonal CD4+Foxp3+ regulatory T cells. The interaction between tolerogenic DC and polyclonal regulatory T cells was important to suppress lupus-like syndrome in mice. Based on this evidence, we hypothesised that regulatory T cell-derived TGF-β can induce an increase in IDO enzyme in DC especially in RA. Various evidence demonstrated an important role for the IDO enzyme in the animal model of arthritis. Criado and colleagues (2009) reported that arthritis induction using immunisation of type II collagen in IDO knockout mice showed a higher incidence of arthritis and exacerbated the severity of arthritis compared to normal mice. By using the IDO inhibitor, 1-methyl-trytophan (1MT), Szanto et al (2007) demonstrated the role of IDO in experimental arthritis using CIA model. Mice immunised with 1MT showed higher incidence of arthritis and experienced more severe arthritis compared to unimmunised mice. Another strategy employed to assess the role of IDO in experimental arthritis was by using gene therapy. Chen and colleagues (2011) showed that intra articular delivery of the IDO gene in rat-collagen induced arthritis can ameliorate arthritis in these rats. These articles showed the role played by IDO in the resolution of arthritis in animal models.

Fallarino et al (2012) described the IDO enzyme as a moonlighting protein in their review as they showed that it can perform multiple biological functions. IDO enzyme is known to catalyse tryptophan, an essential amino acid to kyneurenine and its expression is associated with an immunosuppressive effect on T cell proliferation. Therefore, it is possible that IDO can exert other biological effects. It has been shown by Belladonna and colleagues (2008) that murine tolerogenic CD8+ DC cells can secrete TGF-β and induce IDO expression in immunogenic CD8- DC cells and turn them into tolerogenic DC. TGF-β can induce IDO expression in CD8- DC cells by activation of PI3K/Akt and non-canonical NFκB signalling. Furthermore, Pallotta et al (2011) showed that TGF-β can induce IDO expression and its regulatory phenotype in the murine model of transplantation. The regulatory phenotype in murine pDC was mediated by TGF-β binding to the immunoreceptor tyrosine-based inhibitory (ITIM) motif in pDC leading to the recruitment and activation of phosphatases. The downstream effect of this signalling was the activation of non-canonical activation of the NFκB signalling pathway and the secretion of Type 1 IFN. Type 1 IFN which consists of

IFNα and IFNβ served as a protective factor in RA as reviewed by Crow (2010). She demonstrated the various protective roles of Type 1 IFN especially IFNβ in animal models of arthritis and data from RA patients.

TGF-β binding to ITIM motif can initiate the regulatory phenotype in DC. However, IL-6 concentration is abundant in an RA microenvironment especially in the joints of RA patients. Grohmann et al (2001) showed that IL-6 can impair tolerogenic activity of murine CD8+ DC by reducing the expression of IDO in these cells. Further work by the same group (Pallotta et al, 2010) demonstrated that IL-6 can induce the expression of suppressor of cytokine signalling 3 (SOCS3) in murine DC and bind to another ITIM motif on IDO. Interaction of SOCS3 and ITIM motif in DC activated a cascade of downstream signalling which will lead to the degradation of IDO enzyme in DC. As demonstrated by Wang et al (2014), IL-6 is one of the cytokines needed for Th17 differentiation and Th17 cells have been implicated in the pathogenesis of RA. Sharma et al (2009) reported that *in vitro* differentiation of murine Th17 cells require the presence of IL-6 to convert regulatory T cells to Th17 phenotype. As reported in the previous paragraph, RA patients that received IL-6 inhibition therapy can correct the balance in the ratio of regulatory T cells to Th17 cells. The last two paragraphs demonstrate the mechanisms of IDO action as a signalling protein.

However, the effect of IDO is not only limited to functioning as a signalling protein. IDO can be induced in DC upon cell-to-cell contact with regulatory T cells. Fallarino et al (2003) showed that the engagement of CTLA-4 of murine regulatory T cells with B7 expression or CD80/CD86 expression on DC can induce IDO expression. In addition, Hill et al (2007) reported that IDO expressing DC can expand human regulatory T cells by promoting the proliferation of regulatory T cells *in vitro*. However, Flores-Borja and colleagues (2008) reported that CTLA-4 defects in regulatory T cells of RA patients may contribute to impaired function of regulatory T cells. Further work by Cribbs and co-workers (2014) showed that a fault in the CTLA-4 gene of RA patients is responsible for downregulation of CTLA-4 expression in regulatory T cells from RA patients. They demonstrated that CTLA-4 promoter methylation disabled regulatory T cells from inducing IDO expression in DC thus inflammation persists in RA patients as CTLA-4 had been shown to be important in mediating murine regulatory T cell suppression (Wing et al, 2008). Therefore, it would be

interesting to investigate whether BiP can have a direct effect on CTLA-4 expression on human regulatory T cells.

Previous work by Corrigall et al (2009) demonstrated that human monocyte-derived DCs can induce IDO expression when cultured with BiP and these DCs can convert CD4+CD25- T cells into CD4+CD25+CTLA-4+ regulatory T cells. The ability of DC to induce regulatory T cell development is dependent on the product of tryptophan catabolism, kynurenine. Chen et al (2008) demonstrated that kynurenine can convert human CD4+CD25- T cells to CD4+CD25+Foxp3+ regulatory T cells and this effect was mediated by kynurenine binding to aryl hydrocarbon receptor (Mezrich et al (2010)). Furthermore, kynurenine had been demonstrated to exert an immunosuppressive effect on other immune cells by inhibiting the proliferation of human T cells (Frumento et al (2002) and Terness et al (2002)) and natural killer cells (Frumento et al (2002)). In conclusion, BiP may modulate regulatory and responder T cell functions and further induce the immunoregulatory phenotype as demonstrated in Figure 6.5.

This work added more to the understanding of the immunoregulatory phenotypes that can be induced by soluble BiP on regulatory and responder T cells. Soluble BiP may enhance Foxp3 expression and TGF-β secretion in human regulatory T cells and can induce Foxp3+CD25+ cells and enhanced IL-10 secretion in human responder T cells. As demonstrated by Foo (2011), BiP immunisation in mice can induce BiP-specific cells. These BiP-specific cells were CD4+Foxp3-CTLA-4+PD-1+ cells and can secrete IL-10, TGF-β and soluble CTLA-4. More importantly, these cells are regulatory *in vitro* as demonstrated by their ability to suppress the proliferation of the pathogenic CII cells. Further work by Brownlie et al (2006) showed that BiP-primed cells from immunised mice can increase the secretion of IL-4, IL-5 and IL-10 after re-stimulation with BiP in *in vitro* culture. In addition, BiP-immunised cells adoptively transferred into the CIA model can cure arthritis in the CIA model as measured by the mean severity score (Brownlie et al (2006)). These mechanisms and other immune mechanisms as discussed in Chapter 1 may contribute to the resolution of arthritis in experimental models of arthritis as demonstrated by various studies (Corrigall et al (2001), Brownlie et al (2006), Yoshida et al (2011) and Shields et al (2015)). It is too early to speculate on the efficacy of BiP injection as a treatment for RA as it is being used in Stage I of clinical trials. Therefore, it is essential to compare *in vitro* work performed on

various immune cells from healthy donors (Bodman Smith et al (2003), Corrigall et al (2004), Corrigall et al (2009) and our observation) to the RA patients who received BiP injections once the trial has been completed.

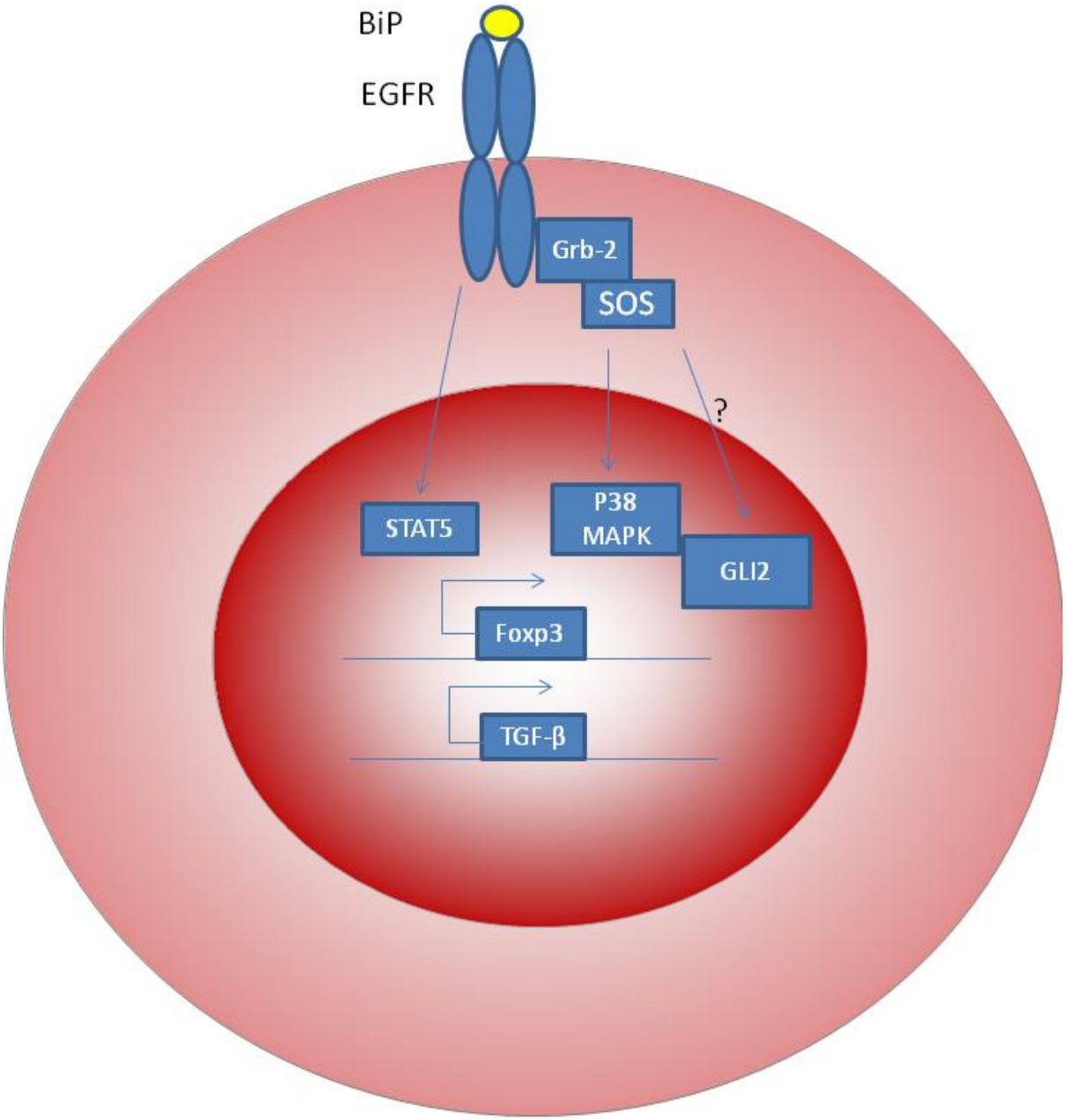


Figure 6.1 Putative BiP signalling in regulatory T cells. BiP binds to EGFR may initiate activation of STAT5 and p38 MAPK. Phosphorylation of STAT5 may lead to an increase in Foxp3 expression while activation of p38MAPK and GLI2 may possibly lead to an increase in TGF-β secretion in regulatory T cells.

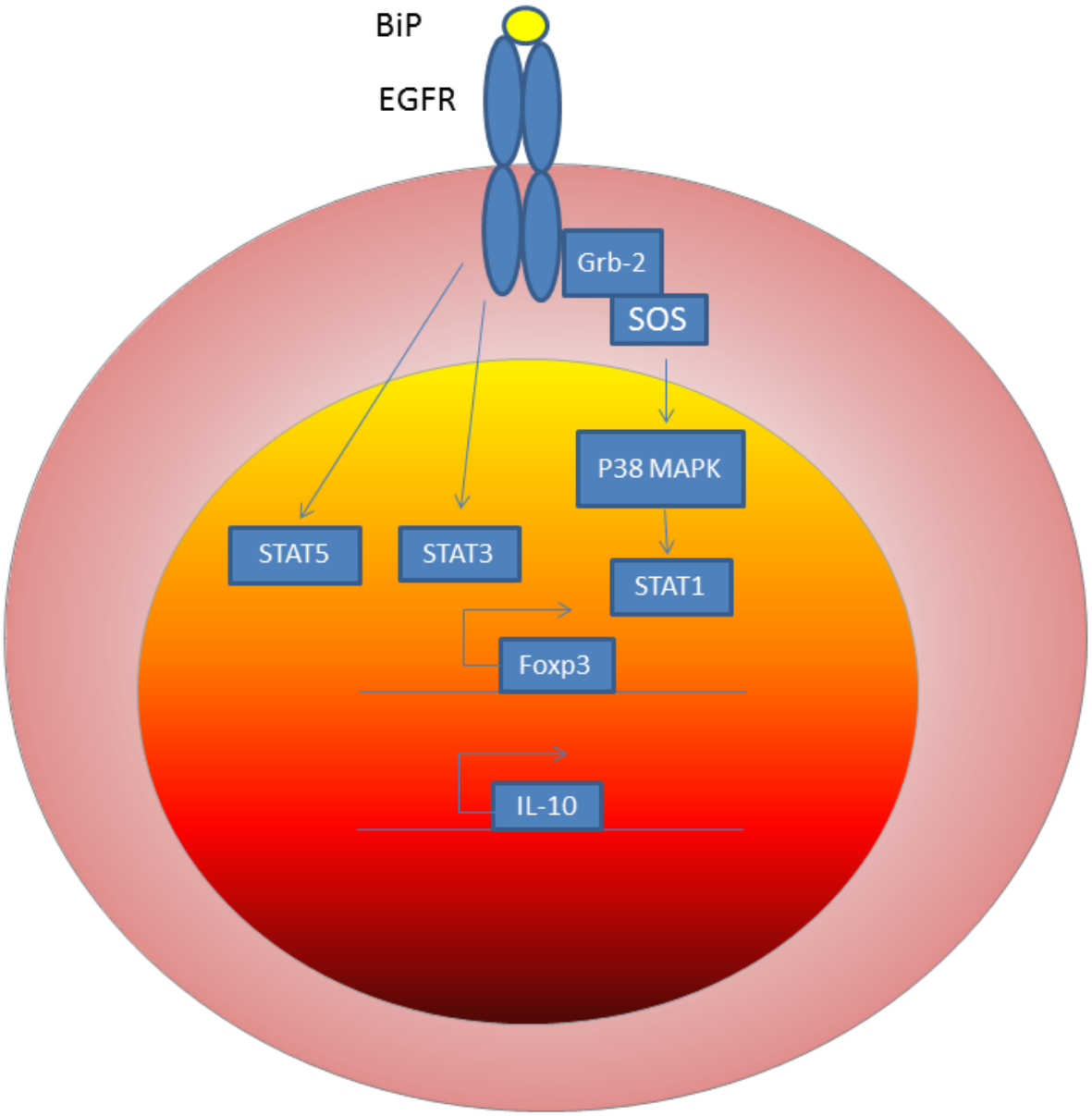


Figure 6.2 Putative BiP signalling in responder T cells. BiP binds to EGFR thus initiating activation of STAT5, STAT3 and p38 MAPK. Phosphorylation of STAT5 leads to enhanced Foxp3 expression while activation of p38MAPK (STAT1) and STAT3 possibly leads to IL-10 secretion in responder T cells.

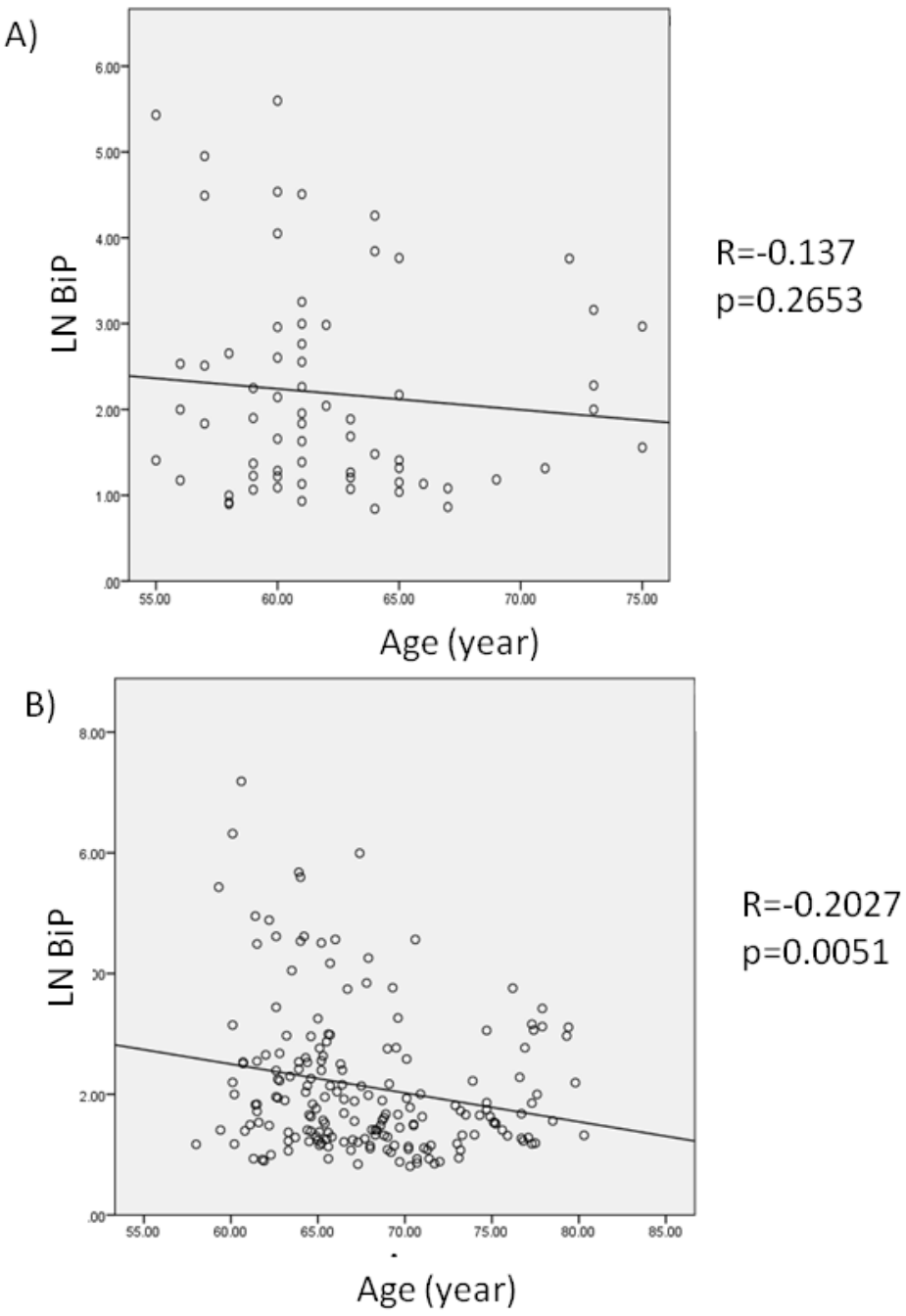


Figure 6.3 Circulating BiP levels correlate with age. Circulating BiP concentrations were measured using ELISA in plasma from the Whitehall II cohort. Each dot represents one donor in the Whitehall II cohort. Panel (A-B) shows a scatter plot of circulating BiP levels against age (n=68) (A) and (n=189) (B). R value represents the correlation coefficient where a positive R value indicates a linear correlation whilst a negative R value demonstrates an inverse correlation. P<0.05 indicates the correlation is statistically significant.

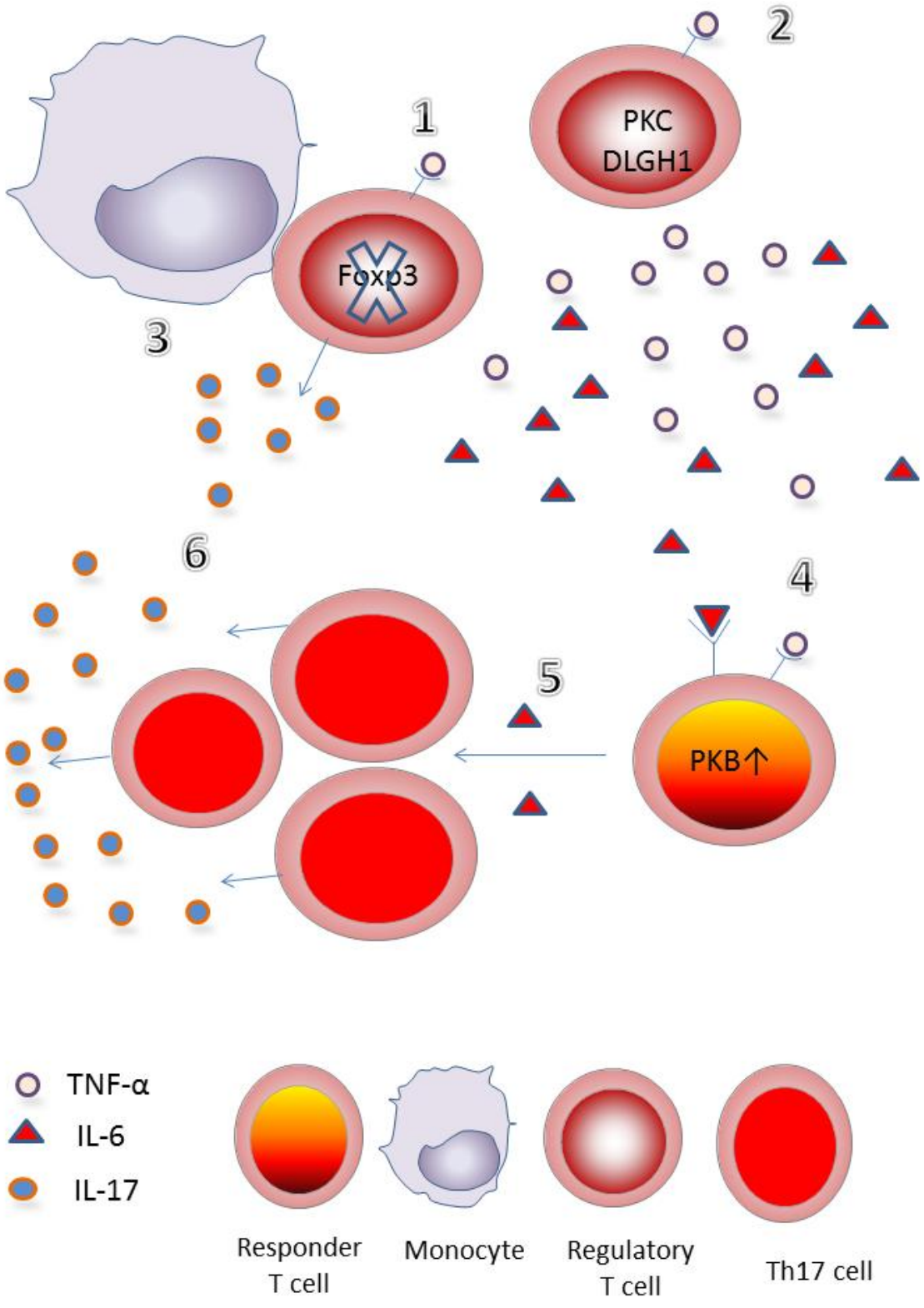
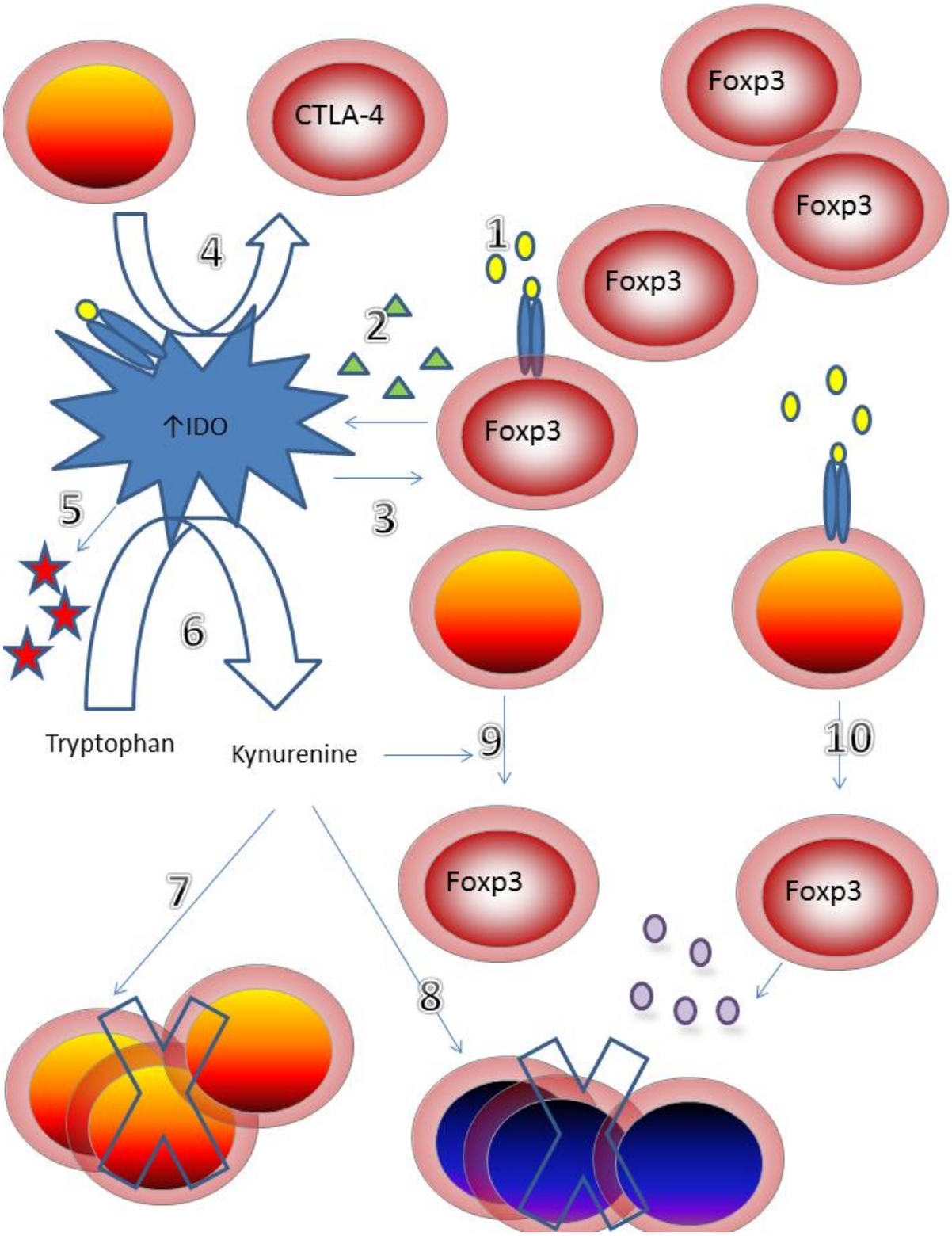

1
2
PKC
DLGH1
Foxp3
3
6
4
5
PKB↑
TNF-α
IL-6
IL-17
Responder
T cell
Monocyte
Regulatory
T cell
Th17 cell

Figure 6.4 Inflammatory milieu influences regulatory and responder T cell phenotype and function. 1 TNF-α ligation on regulatory T cells disrupts phosphorylation of Foxp3 and **2** PKC-θ and DLGH1 role in immunological synapse. **3** Activated monocytes induce regulatory T cells to secrete IL-17. 4 TNF-α and IL-6 ligation to their receptors on responder T cell renders responder T cells hyperactive and resistant to suppression. **5** Responder T cells convert to Th17 cells in the presence of IL-6 and **6** increased Th17 cells leads to imbalance between regulatory T cells and Th17 cells.


CTLA-4
Foxp3
Foxp3
Foxp3
Foxp3
Foxp3
↑IDO
1
2
3
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5
6
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9
10
Tryptophan
Kynurenine

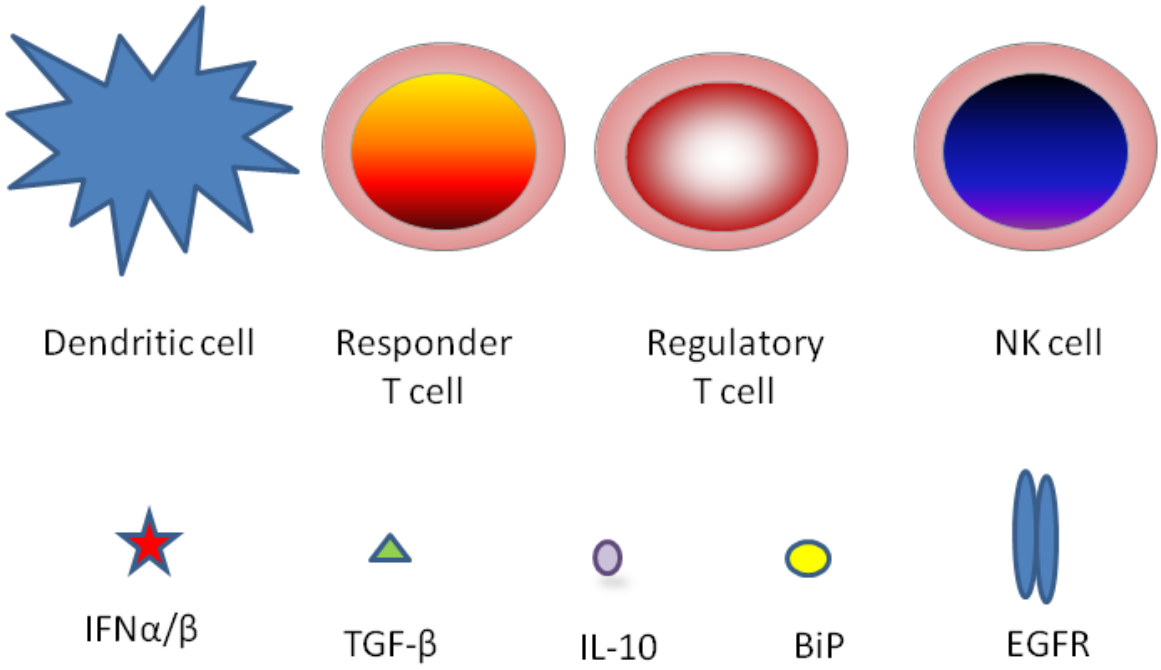

Figure 6.5 How BiP can restore regulatory and responder T cell function 1 BiP ligation on regulatory T cells enhances Foxp3 and induces TGF-β secretion. **2** TGF-β can induce IDO and binds to ITIM motif to activate signalling in IDO to induce tolerance. **3** IDO expression can expand regulatory T cells. **4** BiP can induce tolerogenic DC with increased IDO expression. **5** Tolerogenic DC can induce CTLA-4+ regulatory T cells and can secrete IFNα/β (**6**) and induce catabolism of trytophan to kynurenine (**7**). Kynurenine can induce immunsuppression by inhibiting the proliferation of responder T cells (**8**) and NK cells (**9**) and also convert responder to Foxp3+ regulatory T cells (**10**). In addition, BiP can directly bind to the receptor on responder T cells to induce Foxp3+ regulatory T cells which in turn induce IL-10 to modulate TNF-α.

Chapter 7

Conclusion & Future Work

7.0 Conclusion & Future Work

7.1 Conclusion

The main aim of this project was to investigate the effect of BiP on regulatory T cell frequency and function. Chapter 3 shows that soluble BiP concentration does not correlate with regulatory T cell frequency. However, correlation analysis revealed a linear relationship between two variables. The examples are soluble BiP concentrations correlate with IL-1β, TNF-α and IL-10 concentrations. Therefore, although soluble BiP concentration has no relationship with regulatory T cell frequency, it is possible that soluble BiP may affect or modulate regulatory T cell function.

Therefore, the aim of Chapter 4 was to investigate the effect of BiP on regulatory T cell phenotype and function. Two hours of BiP pre-treatment does not modulate regulatory T cell function as does HSP60. However, regulatory T cells cultured with BiP for 4 days may enhance Foxp3 expression and increase TGF-β secretion but these effects were not translated to an enhanced suppressive capacity of regulatory T cells on responder T cells.

Chapter 5 was based on the initial observation in Chapter 4 where responder T cells cultured in the presence of anti-CD3/CD28 beads with BiP can significantly reduce their proliferation. Phenotypic analysis revealed an increase in Foxp3+CD25+ cells within unstimulated responder T cells cultured with BiP. These cells can secrete IL-10 and can downregulate TNF-α secretion from autologous responder T cells in co-culture.

In conclusion, BiP may modulate regulatory T cell phenotype and can affect responder T cell phenotype and function.

7.2 Future Works

To further investigate the effect of BiP on regulatory and responder T cells, future work will mainly focus on following up Chapter 4 and Chapter 5. The list of the future works are as follows:-

Chapter 4 – The effect of soluble BiP on regulatory T cell phenotype and function:-

- To confirm Foxp3 expression and TGF-β secretion by 4 days of BiP treatment on regulatory T cells by measuring using Foxp3 and TGF-β mRNA using reverse transcription polymerase chain reaction (RT-PCR)
- Investigating the effect of soluble BiP on CTLA-4 expression in regulatory T cells as CTLA-4 expression in regulatory T cells of RA patients was decreased
- Investigating the effect of BiP-treated regulatory T cells on responder T cell proliferation in the presence of APC (monocytes)
- Investigating the effect of BiP-treated regulatory T cells on IDO expression in DC

Chapter 5 – The effect of soluble BiP on responder T cell phenotype and function:-

- Investigate the surface markers associated with BiP-induced Foxp3+CD25+ cells before these cells can be sorted to assess their suppressive capacity
- To confirm that TNF-α downregulation by BiP-induced Foxp3+CD25+ cells is because of IL-10 secretion by using neutralising antibodies towards IL-10 in the co-culture

Chapter 8

References

8.0 References

AALBERSE, J. A., KAPITEIN, B., DE ROOCK, S., KLEIN, M. R., DE JAGER, W., VAN DER ZEE, R., HOEKSTRA, M. O., VAN WIJK, F. & PRAKKEN, B. J. 2011. Cord Blood CD4 T Cells Respond to Self Heat Shock Protein 60 (HSP60). *PLoS One,* 6**,** e24119.

ABBAS, A. K., BENOIST, C., BLUESTONE, J. A., CAMPBELL, D. J., GHOSH, S., HORI, S., JIANG, S., KUCHROO, V. K., MATHIS, D., RONCAROLO, M. G., RUDENSKY, A., SAKAGUCHI, S., SHEVACH, E. M., VIGNALI, D. A. & ZIEGLER, S. F. 2013. Regulatory T cells: recommendations to simplify the nomenclature. *Nature immunology,* 14**,** 307-8.

AHIMA, R. S. 2008. Revisiting leptin's role in obesity and weight loss. *The Journal of Clinical Investigation,* 118**,** 2380-2383.

AIT-OUFELLA, H., SALOMON, B. L., POTTEAUX, S., ROBERTSON, A. K., GOURDY, P., ZOLL, J., MERVAL, R., ESPOSITO, B., COHEN, J. L., FISSON, S., FLAVELL, R. A., HANSSON, G. K., KLATZMANN, D., TEDGUI, A. & MALLAT, Z. 2006. Natural regulatory T cells control the development of atherosclerosis in mice. *Nature medicine,* 12**,** 178-80.

AKIMOVA, T., BEIER, U. H., WANG, L., LEVINE, M. H. & HANCOCK, W. W. 2011. Helios expression is a marker of T cell activation and proliferation. *PLoS One,* 6**,** e24226.

ALLAN, S. E., CROME, S. Q., CRELLIN, N. K., PASSERINI, L., STEINER, T. S., BACCHETTA, R., RONCAROLO, M. G. & LEVINGS, M. K. 2007. Activation-induced FOXP3 in human T effector cells does not suppress proliferation or cytokine production. *International immunology,* 19**,** 345-54.

ALMGREN, M., ATKINSON, R., HE, J., HILDING, A., HAGMAN, E., WOLK, A., THORELL, A., MARCUS, C., NÄSLUND, E., ÖSTENSON, C.-G., SCHALLING, M. & LAVEBRATT, C. 2012. Adenovirus-36 Is Associated with Obesity in Children and Adults in Sweden as Determined by Rapid ELISA. *PLoS One,* 7**,** e41652.

AMMIRATI, E., CIANFLONE, D., BANFI, M., VECCHIO, V., PALINI, A., DE METRIO, M., MARENZI, G., PANCIROLI, C., TUMMINELLO, G., ANZUINI, A., PALLOSHI, A., GRIGORE, L., GARLASCHELLI, K., TRAMONTANA, S., TAVANO, D., AIROLDI, F., MANFREDI, A. A., CATAPANO, A. L. & NORATA, G. D. 2010. Circulating CD4+CD25hiCD127lo regulatory T-Cell levels do not reflect the extent or severity of carotid and coronary atherosclerosis. *Arterioscler Thromb Vasc Biol,* 30**,** 1832-41.

ANDERSSON, J., TRAN, D. Q., PESU, M., DAVIDSON, T. S., RAMSEY, H., O'SHEA, J. J. & SHEVACH, E. M. 2008. CD4+ FoxP3+ regulatory T cells confer infectious tolerance in a TGF-beta-dependent manner. *The Journal of experimental medicine,* 205**,** 1975-81.

ANDOLFI, G., FOUSTERI, G., ROSSETTI, M., MAGNANI, C. F., JOFRA, T., LOCAFARO, G., BONDANZA, A., GREGORI, S. & RONCAROLO, M. G. 2012. Enforced IL-10 expression confers type 1 regulatory T cell (Tr1) phenotype and function to human CD4(+) T cells. *Mol Ther,* 20**,** 1778-90.

ANNUNZIATO, F., COSMI, L., LIOTTA, F., LAZZERI, E., MANETTI, R., VANINI, V., ROMAGNANI, P., MAGGI, E. & ROMAGNANI, S. 2002. Phenotype, localization, and mechanism of suppression of CD4(+)CD25(+) human thymocytes. *The Journal of experimental medicine,* 196**,** 379-87.

ASSEMAN, C., MAUZE, S., LEACH, M. W., COFFMAN, R. L. & POWRIE, F. 1999. An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *The Journal of experimental medicine,* 190**,** 995-1004.

AZIZ, N., NISHANIAN, P., MITSUYASU, R., DETELS, R. & FAHEY, J. L. 1999. Variables that affect assays for plasma cytokines and soluble activation markers. *Clin Diagn Lab Immunol,* 6**,** 89-95.

BACHMAIER, K., NEU, N., DE LA MAZA, L. M., PAL, S., HESSEL, A. & PENNINGER, J. M. 1999. Chlamydia infections and heart disease linked through antigenic mimicry. *Science,* 283**,** 1335-9.

BAECHER-ALLAN, C., BROWN, J. A., FREEMAN, G. J. & HAFLER, D. A. 2001. CD4+CD25high regulatory cells in human peripheral blood. *Journal of immunology,* 167**,** 1245-53.

BARKER, R. N., ERWIG, L. P., HILL, K. S., DEVINE, A., PEARCE, W. P. & REES, A. J. 2002. Antigen presentation by macrophages is enhanced by the uptake of necrotic, but not apoptotic, cells. *Clinical and experimental immunology,* 127**,** 220-5.

BEIER, U. H., WANG, L., HAN, R., AKIMOVA, T., LIU, Y. & HANCOCK, W. W. 2012. Histone deacetylases 6 and 9 and sirtuin-1 control Foxp3+ regulatory T cell function through shared and isoform-specific mechanisms. *Science signaling,* 5**,** ra45.

BELLADONNA, M. L., VOLPI, C., BIANCHI, R., VACCA, C., ORABONA, C., PALLOTTA, M. T., BOON, L., GIZZI, S., FIORETTI, M. C., GROHMANN, U. & PUCCETTI, P. 2008. Cutting edge: Autocrine TGF-beta sustains default tolerogenesis by IDO-competent dendritic cells. *Journal of immunology,* 181**,** 5194-8.

BERGER, C. L., DONG, Z., HANLON, D., BISACCIA, E. & EDELSON, R. L. 1997. A lymphocyte cell surface heat shock protein homologous to the endoplasmic reticulum chaperone, immunoglobulin heavy chain binding protein BIP. *Int J Cancer,* 71**,** 1077-85.

BHATTACHARJEE, G., AHAMED, J., PEDERSEN, B., EL-SHEIKH, A., MACKMAN, N., RUF, W., LIU, C. & EDGINGTON, T. S. 2005. Regulation of tissue factor--mediated initiation of the coagulation cascade by cell surface grp78. *Arterioscler Thromb Vasc Biol,* 25**,** 1737-43.

BIREBENT, B., LORHO, R., LECHARTIER, H., DE GUIBERT, S., ALIZADEH, M., VU, N., BEAUPLET, A., ROBILLARD, N. & SEMANA, G. 2004. Suppressive properties of human CD4+CD25+ regulatory T cells are dependent on CTLA-4 expression. *European journal of immunology,* 34**,** 3485-96.

BISHARA, J., FARAH, R., MOGRABI, J., KHALAILA, W., ABU-ELHEJA, O., MAHAMID, M. & NSEIR, W. 2013. Obesity as a risk factor for Clostridium difficile infection. *Clin Infect Dis,* 57**,** 489-93.

BLASS, S., SPECKER, C., LAKOMEK, H. J., SCHNEIDER, E. M. & SCHWOCHAU, M. 1995. Novel 68 kDa autoantigen detected by rheumatoid arthritis specific antibodies. *Annals of the rheumatic diseases,* 54**,** 355-60.

BLASS, S., UNION, A., RAYMACKERS, J., SCHUMANN, F., UNGETHUM, U., MULLER-STEINBACH, S., DE KEYSER, F., ENGEL, J. M. & BURMESTER, G. R. 2001. The stress protein BiP is overexpressed and is a major B and T cell target in rheumatoid arthritis. *Arthritis and rheumatism,* 44**,** 761-71.

BODMAN-SMITH, M. D., CORRIGALL, V. M., KEMENY, D. M. & PANAYI, G. S. 2003. BiP, a putative autoantigen in rheumatoid arthritis, stimulates IL-10-producing CD8-positive T cells from normal individuals. *Rheumatology,* 42**,** 637-44.

BOELAERT, K., NEWBY, P. R., SIMMONDS, M. J., HOLDER, R. L., CARR-SMITH, J. D., HEWARD, J. M., MANJI, N., ALLAHABADIA, A., ARMITAGE, M., CHATTERJEE, K. V., LAZARUS, J. H., PEARCE, S. H., VAIDYA, B., GOUGH, S. C. & FRANKLYN, J. A. 2010. Prevalence and relative risk of other autoimmune diseases in subjects with autoimmune thyroid disease. *Am J Med,* 123**,** 183 e1-9.

BOGAERT, S., DE VOS, M., OLIEVIER, K., PEETERS, H., ELEWAUT, D., LAMBRECHT, B., POULIOT, P. & LAUKENS, D. 2011. Involvement of endoplasmic reticulum stress in

inflammatory bowel disease: a different implication for colonic and ileal disease? *PLoS One,* 6**,** e25589.

BOGDAN, C., PAIK, J., VODOVOTZ, Y. & NATHAN, C. 1992. Contrasting mechanisms for suppression of macrophage cytokine release by transforming growth factor-beta and interleukin-10. *The Journal of biological chemistry,* 267**,** 23301-8.

BRENNAN, F. M. & MCINNES, I. B. 2008. Evidence that cytokines play a role in rheumatoid arthritis. *Journal of Clinical Investigation,* 118**,** 3537-45.

BROCCHIERI, L., CONWAY DE MACARIO, E. & MACARIO, A. J. 2008. hsp70 genes in the human genome: Conservation and differentiation patterns predict a wide array of overlapping and specialized functions. *BMC Evol Biol,* 8**,** 19.

BROWNLIE, R. J. 2004. *The immunotherapy of collagen induced arthritis by the stress protein BIP.* PhD, King's College London.

BROWNLIE, R. J., MYERS, L. K., WOOLEY, P. H., CORRIGALL, V. M., BODMAN-SMITH, M. D., PANAYI, G. S. & THOMPSON, S. J. 2006. Treatment of murine collagen-induced arthritis by the stress protein BiP via interleukin-4-producing regulatory T cells: a novel function for an ancient protein. *Arthritis and rheumatism,* 54**,** 854-63.

BRUNKOW, M. E., JEFFERY, E. W., HJERRILD, K. A., PAEPER, B., CLARK, L. B., YASAYKO, S. A., WILKINSON, J. E., GALAS, D., ZIEGLER, S. F. & RAMSDELL, F. 2001. Disruption of a new forkhead/winged-helix protein, scurfin, results in the fatal lymphoproliferative disorder of the scurfy mouse. *Nat Genet,* 27**,** 68-73.

BUCHKOVICH, N. J., YU, Y., PIERCIEY, F. J., JR. & ALWINE, J. C. 2010. Human cytomegalovirus induces the endoplasmic reticulum chaperone BiP through increased transcription and activation of translation by using the BiP internal ribosome entry site. *J Virol,* 84**,** 11479-86.

BURCHILL, M. A., YANG, J., VOGTENHUBER, C., BLAZAR, B. R. & FARRAR, M. A. 2007. IL-2 Receptor β-Dependent STAT5 Activation Is Required for the Development of Foxp3+ Regulatory T Cells. *The Journal of Immunology,* 178**,** 280-290.

CAAMANO, J. & HUNTER, C. A. 2002. NF-kappaB family of transcription factors: central regulators of innate and adaptive immune functions. *Clin Microbiol Rev,* 15**,** 414-29.

CALLARD, R. E. G., A.J.H. (ed.) 1994. *Cytokine receptor superfamilies,* San Diego: Academic Press Inc.

CARR, J. J. & DING, J. 2009. Response to “Epicardial and Pericardial Fat: Close, but Very Different”. *Obesity,* 17**,** 626-627.

CARTER, L. L. & SWAIN, S. L. 1997. Single cell analyses of cytokine production. *Current opinion in immunology,* 9**,** 177-82.

CEDERBOM, L., HALL, H. & IVARS, F. 2000. CD4+CD25+ regulatory T cells down-regulate co-stimulatory molecules on antigen-presenting cells. *European journal of immunology,* 30**,** 1538-43.

CHAN, G., NOGALSKI, M. T. & YUROCHKO, A. D. 2009. Activation of EGFR on monocytes is required for human cytomegalovirus entry and mediates cellular motility. *Proceedings of the National Academy of Sciences of the United States of America,* 106**,** 22369-74.

CHANG, J. S., OCVIRK, S., BERGER, E., KISLING, S., BINDER, U., SKERRA, A., LEE, A. S. & HALLER, D. 2012. Endoplasmic reticulum stress response promotes cytotoxic phenotype of CD8alphabeta+ intraepithelial lymphocytes in a mouse model for Crohn's disease-like ileitis. *Journal of immunology,* 189**,** 1510-20.

CHEN, S. Y., WU, C. L., LAI, M. D., LIN, C. C., YO, Y. T., JOU, I. M., LEE, C. H., WENG, C. T., SHIAU, A. L. & WANG, C. R. 2011. Amelioration of rat collagen-induced arthritis through CD4+ T cells apoptosis and synovial interleukin-17 reduction by indoleamine 2,3-dioxygenase gene therapy. *Hum Gene Ther,* 22**,** 145-54.

CHEN, W., JIN, W., HARDEGEN, N., LEI, K. J., LI, L., MARINOS, N., MCGRADY, G. & WAHL, S. M. 2003. Conversion of peripheral CD4+CD25- naive T cells to CD4+CD25+ regulatory T cells by TGF-beta induction of transcription factor Foxp3. *The Journal of experimental medicine,* 198**,** 1875-86.

CHEN, W., LIANG, X., PETERSON, A. J., MUNN, D. H. & BLAZAR, B. R. 2008. The indoleamine 2,3-dioxygenase pathway is essential for human plasmacytoid dendritic cell-induced adaptive T regulatory cell generation. *Journal of immunology,* 181**,** 5396-404.

CORRIGALL, V. M., BODMAN-SMITH, M. D., BRUNST, M., CORNELL, H. & PANAYI, G. S. 2004. Inhibition of antigen-presenting cell function and stimulation of human peripheral blood mononuclear cells to express an antiinflammatory cytokine profile by the stress protein BiP: relevance to the treatment of inflammatory arthritis. *Arthritis and rheumatism,* 50**,** 1164-71.

CORRIGALL, V. M., BODMAN-SMITH, M. D., FIFE, M. S., CANAS, B., MYERS, L. K., WOOLEY, P., SOH, C., STAINES, N. A., PAPPIN, D. J., BERLO, S. E., VAN EDEN, W., VAN DER ZEE, R., LANCHBURY, J. S. & PANAYI, G. S. 2001. The human endoplasmic reticulum molecular chaperone BiP is an autoantigen for rheumatoid arthritis and prevents the induction of experimental arthritis. *Journal of immunology,* 166**,** 1492-8.

CORRIGALL, V. M., VITTECOQ, O. & PANAYI, G. S. 2009. Binding immunoglobulin protein-treated peripheral blood monocyte-derived dendritic cells are refractory to maturation and induce regulatory T-cell development. *Immunology,* 128**,** 218-26.

CRIADO, G., SIMELYTE, E., INGLIS, J. J., ESSEX, D. & WILLIAMS, R. O. 2009. Indoleamine 2,3 dioxygenase-mediated tryptophan catabolism regulates accumulation of Th1/Th17 cells in the joint in collagen-induced arthritis. *Arthritis and rheumatism,* 60**,** 1342-51.

CRIBBS, A. P., KENNEDY, A., PENN, H., READ, J. E., AMJADI, P., GREEN, P., SYED, K., MANKA, S. W., BRENNAN, F. M., GREGORY, B. & WILLIAMS, R. O. 2014. Treg cell function in rheumatoid arthritis is compromised by ctla-4 promoter methylation resulting in a failure to activate the indoleamine 2,3-dioxygenase pathway. *Arthritis Rheumatol,* 66**,** 2344-54.

CROW, M. K. 2010. Type I interferon in organ-targeted autoimmune and inflammatory diseases. *Arthritis research & therapy,* 12 Suppl 1**,** S5.

DAS, I., PNG, C. W., OANCEA, I., HASNAIN, S. Z., LOURIE, R., PROCTOR, M., ERI, R. D., SHENG, Y., CRANE, D. I., FLORIN, T. H. & MCGUCKIN, M. A. 2013. Glucocorticoids alleviate intestinal ER stress by enhancing protein folding and degradation of misfolded proteins. *The Journal of experimental medicine,* 210**,** 1201-16.

DAUGAARD, M., ROHDE, M. & JAATTELA, M. 2007. The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. *FEBS letters,* 581**,** 3702-10.

DE JAGER, W., BOURCIER, K., RIJKERS, G. T., PRAKKEN, B. J. & SEYFERT-MARGOLIS, V. 2009. Prerequisites for cytokine measurements in clinical trials with multiplex immunoassays. *BMC immunology,* 10**,** 52.

DE JAGER, W., PRAKKEN, B. J., BIJLSMA, J. W., KUIS, W. & RIJKERS, G. T. 2005. Improved multiplex immunoassay performance in human plasma and synovial fluid following removal of interfering heterophilic antibodies. *J Immunol Methods,* 300**,** 124-35.

DE KLEER, I., VERCOULEN, Y., KLEIN, M., MEERDING, J., ALBANI, S., VAN DER ZEE, R., SAWITZKI, B., HAMANN, A., KUIS, W. & PRAKKEN, B. 2010. CD30 discriminates heat shock protein 60-induced FOXP3+ CD4+ T cells with a regulatory phenotype. *Journal of immunology,* 185**,** 2071-9.

DE ROSA, V., PROCACCINI, C., CALI, G., PIROZZI, G., FONTANA, S., ZAPPACOSTA, S., LA CAVA, A. & MATARESE, G. 2007. A key role of leptin in the control of regulatory T cell proliferation. *Immunity,* 26**,** 241-55.

DELPINO, A. & CASTELLI, M. 2002. The 78 kDa glucose-regulated protein (GRP78/BIP) is expressed on the cell membrane, is released into cell culture medium and is also present in human peripheral circulation. *Biosci Rep,* 22**,** 407-20.

DEMPSEY, N. C., LEONI, F., IRELAND, H. E., HOYLE, C. & WILLIAMS, J. H. 2010. Differential heat shock protein localization in chronic lymphocytic leukemia. *Journal of leukocyte biology,* 87**,** 467-76.

DEURING, J. J., DE HAAR, C., KOELEWIJN, C. L., KUIPERS, E. J., PEPPELENBOSCH, M. P. & VAN DER WOUDE, C. J. 2012. Absence of ABCG2-mediated mucosal detoxification in patients with active inflammatory bowel disease is due to impeded protein folding. *Biochem J,* 441**,** 87-93.

DIECKMANN, D., BRUETT, C. H., PLOETTNER, H., LUTZ, M. B. & SCHULER, G. 2002. Human CD4(+)CD25(+) regulatory, contact-dependent T cells induce interleukin 10-producing, contact-independent type 1-like regulatory T cells [corrected]. *The Journal of experimental medicine,* 196**,** 247-53.

DIECKMANN, D., PLOTTNER, H., BERCHTOLD, S., BERGER, T. & SCHULER, G. 2001. Ex vivo isolation and characterization of CD4(+)CD25(+) T cells with regulatory properties from human blood. *The Journal of experimental medicine,* 193**,** 1303-10.

DIETRICH, T., HUCKO, T., SCHNEEMANN, C., NEUMANN, M., MENRAD, A., WILLUDA, J., ATROTT, K., STIBENZ, D., FLECK, E., GRAF, K. & MENSSEN, H. D. 2012. Local delivery of IL-2 reduces atherosclerosis via expansion of regulatory T cells. *Atherosclerosis,* 220**,** 329-336.

DOMBRECHT, E. J., AERTS, N. E., SCHUERWEGH, A. J., HAGENDORENS, M. M., EBO, D. G., VAN OFFEL, J. F., BRIDTS, C. H., STEVENS, W. J. & DE CLERCK, L. S. 2006. Influence of anti-tumor necrosis factor therapy (Adalimumab) on regulatory T cells and dendritic cells in rheumatoid arthritis. *Clin Exp Rheumatol,* 24**,** 31-7.

DONG, W., LI, X., FENG, Y., FAN, C., CHEN, Z. & ZHU, P. 2009. The differential expressions of 78-kDa glucose-regulated protein of infiltrating plasma cells in peripheral joints with the histopathological variants of rheumatoid synovitis. *Arthritis research & therapy,* 11**,** R4.

EHLERS, S. & SMITH, K. A. 1991. Differentiation of T cell lymphokine gene expression: the in vitro acquisition of T cell memory. *The Journal of experimental medicine,* 173**,** 25-36.

EHRENSTEIN, M. R., EVANS, J. G., SINGH, A., MOORE, S., WARNES, G., ISENBERG, D. A. & MAURI, C. 2004. Compromised function of regulatory T cells in rheumatoid arthritis and reversal by anti-TNFalpha therapy. *The Journal of experimental medicine,* 200**,** 277-85.

ENGIN, F., YERMALOVICH, A., NGUYEN, T., HUMMASTI, S., FU, W., EIZIRIK, D. L., MATHIS, D. & HOTAMISLIGIL, G. S. 2013. Restoration of the Unfolded Protein Response in Pancreatic β Cells Protects Mice Against Type 1 Diabetes. *Science Translational Medicine,* 5**,** 211ra156.

ESQUERRE, M., TAUZIN, B., GUIRAUD, M., MULLER, S., SAOUDI, A. & VALITUTTI, S. 2008. Human regulatory T cells inhibit polarization of T helper cells toward antigen-presenting cells via a TGF-beta-dependent mechanism. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 2550-5.

EVANS, H. G., ROOSTALU, U., WALTER, G. J., GULLICK, N. J., FREDERIKSEN, K. S., ROBERTS, C. A., SUMNER, J., BAETEN, D. L., GERWIEN, J. G., COPE, A. P., GEISSMANN, F., KIRKHAM, B. W. & TAAMS, L. S. 2014. TNF-alpha blockade induces IL-10 expression in human CD4+ T cells. *Nat Commun,* 5**,** 3199.

FABBRINI, E., CELLA, M., MCCARTNEY, S. A., FUCHS, A., ABUMRAD, N. A., PIETKA, T. A., CHEN, Z., FINCK, B. N., HAN, D. H., MAGKOS, F., CONTE, C., BRADLEY, D., FRATERRIGO, G., EAGON, J. C., PATTERSON, B. W., COLONNA, M. & KLEIN, S. 2013. Association between specific adipose tissue CD4+ T-cell populations and insulin resistance in obese individuals. *Gastroenterology,* 145**,** 366-74 e1-3.

FALLARINO, F., GROHMANN, U., HWANG, K. W., ORABONA, C., VACCA, C., BIANCHI, R., BELLADONNA, M. L., FIORETTI, M. C., ALEGRE, M. L. & PUCCETTI, P. 2003. Modulation of tryptophan catabolism by regulatory T cells. *Nature immunology,* 4**,** 1206-12.

FALLARINO, F., GROHMANN, U. & PUCCETTI, P. 2012. Indoleamine 2,3-dioxygenase: from catalyst to signaling function. *European journal of immunology,* 42**,** 1932-7.

FANTINI, M. C., BECKER, C., MONTELEONE, G., PALLONE, F., GALLE, P. R. & NEURATH, M. F. 2004. Cutting edge: TGF-beta induces a regulatory phenotype in CD4+CD25- T cells through Foxp3 induction and down-regulation of Smad7. *Journal of immunology,* 172**,** 5149-53.

FEAVER, R. E., HASTINGS, N. E., PRYOR, A. & BLACKMAN, B. R. 2008. GRP78 Upregulation by Atheroprone Shear Stress Via p38-, α2β1-Dependent Mechanism in Endothelial Cells. *Arteriosclerosis, Thrombosis, and Vascular Biology,* 28**,** 1534-1541.

FERRARO, A., D'ALISE, A. M., RAJ, T., ASINOVSKI, N., PHILLIPS, R., ERGUN, A., REPLOGLE, J. M., BERNIER, A., LAFFEL, L., STRANGER, B. E., DE JAGER, P. L., MATHIS, D. & BENOIST, C. 2014. Interindividual variation in human T regulatory cells. *Proceedings of the National Academy of Sciences of the United States of America,* 111**,** E1111-20.

FLORES-BORJA, F., JURY, E. C., MAURI, C. & EHRENSTEIN, M. R. 2008. Defects in CTLA-4 are associated with abnormal regulatory T cell function in rheumatoid arthritis. *Proceedings of the National Academy of Sciences of the United States of America,* 105**,** 19396-401.

FONTENOT, J. D., DOOLEY, J. L., FARR, A. G. & RUDENSKY, A. Y. 2005. Developmental regulation of Foxp3 expression during ontogeny. *The Journal of experimental medicine,* 202**,** 901-6.

FOO, S. 2011. *Characterisation of BiP-induced regulatory T cells in experimental arthritis.* MSc Immunology, King's College London.

FRANCO, A., ALMANZA, G., BURNS, J. C., WHEELER, M. & ZANETTI, M. 2010. Endoplasmic reticulum stress drives a regulatory phenotype in human T-cell clones. *Cell Immunol,* 266**,** 1-6.

FRUMENTO, G., ROTONDO, R., TONETTI, M., DAMONTE, G., BENATTI, U. & FERRARA, G. B. 2002. Tryptophan-derived catabolites are responsible for inhibition of T and natural killer cell proliferation induced by indoleamine 2,3-dioxygenase. *The Journal of experimental medicine,* 196**,** 459-68.

FURLER, R. L. & UITTENBOGAART, C. H. 2012. GLI2 regulates TGF-beta1 in human CD4+ T cells: implications in cancer and HIV pathogenesis. *PLoS One,* 7**,** e40874.

GEIJTENBEEK, T. B. & GRINGHUIS, S. I. 2009. Signalling through C-type lectin receptors: shaping immune responses. *Nature reviews. Immunology,* 9**,** 465-79.

GOMEZ-PUERTA, J. A., CELIS, R., HERNANDEZ, M. V., RUIZ-ESQUIDE, V., RAMIREZ, J., HARO, I., CANETE, J. D. & SANMARTI, R. 2013. Differences in synovial fluid cytokine levels but not in synovial tissue cell infiltrate between anti-citrullinated peptide/protein antibody-positive and -negative rheumatoid arthritis patients. *Arthritis research & therapy,* 15**,** R182.

GRAINGER, D. J., MOSEDALE, D. E. & METCALFE, J. C. 2000. TGF-beta in blood: a complex problem. *Cytokine Growth Factor Rev,* 11**,** 133-45.

GREIF, M., VON ZIEGLER, F., WAKILI, R., TITTUS, J., BECKER, C., HELBIG, S., LAUBENDER, R. P., SCHWARZ, W., D'ANASTASI, M., SCHENZLE, J., LEBER, A. W. & BECKER, A. 2013. Increased pericardial adipose tissue is correlated with atrial fibrillation and left atrial dilatation. *Clin Res Cardiol,* 102**,** 555-62.

GROHMANN, U., FALLARINO, F., BIANCHI, R., BELLADONNA, M. L., VACCA, C., ORABONA, C., UYTTENHOVE, C., FIORETTI, M. C. & PUCCETTI, P. 2001. IL-6 inhibits the tolerogenic function of CD8 alpha+ dendritic cells expressing indoleamine 2,3-dioxygenase. *Journal of immunology,* 167**,** 708-14.

GUPTA, S. & KNOWLTON, A. A. 2007. HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway. *Am J Physiol Heart Circ Physiol,* 292**,** H3052-6.

HAAS, I. G. & WABL, M. 1983. Immunoglobulin heavy chain binding protein. *Nature,* 306**,** 387-9.

HAN, J. M. & LEVINGS, M. K. 2013. Immune regulation in obesity-associated adipose inflammation. *Journal of immunology,* 191**,** 527-32.

HAN, Y., YANG, Y., CHEN, Z., JIANG, Z., GU, Y., LIU, Y., XU, S., LIN, C., PAN, Z., ZHOU, W. & CAO, X. 2014. Human hepatocellular carcinoma-infiltrating CD4(+)CD69(+)Foxp3(-) regulatory T cell suppresses T cell response via membrane-bound TGF-beta1. *J Mol Med (Berl),* 92**,** 539-50.

HAO, X., YAO, A., GONG, J., ZHU, W., LI, N. & LI, J. 2012. Berberine ameliorates pro-inflammatory cytokine-induced endoplasmic reticulum stress in human intestinal epithelial cells in vitro. *Inflammation,* 35**,** 841-9.

HAUFE, S., HAUG, M., SCHEPP, C., KUEMMERLE-DESCHNER, J., HANSMANN, S., RIEBER, N., TZARIBACHEV, N., HOSPACH, T., MAIER, J., DANNECKER, G. E. & HOLZER, U. 2011.

Impaired suppression of synovial fluid CD4+CD25- T cells from patients with juvenile idiopathic arthritis by CD4+CD25+ Treg cells. *Arthritis and rheumatism,* 63**,** 3153-62.

HAURA, E. B., TURKSON, J. & JOVE, R. 2005. Mechanisms of disease: Insights into the emerging role of signal transducers and activators of transcription in cancer. *Nat Clin Pract Oncol,* 2**,** 315-24.

HAZE, K., YOSHIDA, H., YANAGI, H., YURA, T. & MORI, K. 1999. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell,* 10**,** 3787-99.

HERBIN, O., AIT-OUFELLA, H., YU, W., FREDRIKSON, G. N., AUBIER, B., PEREZ, N., BARATEAU, V., NILSSON, J., TEDGUI, A. & MALLAT, Z. 2012. Regulatory T-cell response to apolipoprotein B100-derived peptides reduces the development and progression of atherosclerosis in mice. *Arterioscler Thromb Vasc Biol,* 32**,** 605-12.

HERRATH, J., CHEMIN, K., ALBRECHT, I., CATRINA, A. I. & MALMSTROM, V. 2014. Surface expression of CD39 identifies an enriched Treg-cell subset in the rheumatic joint, which does not suppress IL-17A secretion. *European journal of immunology,* 44**,** 2979-89.

HILL, M., TANGUY-ROYER, S., ROYER, P., CHAUVEAU, C., ASGHAR, K., TESSON, L., LAVAINNE, F., REMY, S., BRION, R., HUBERT, F. X., HESLAN, M., RIMBERT, M., BERTHELOT, L., MOFFETT, J. R., JOSIEN, R., GREGOIRE, M. & ANEGON, I. 2007. IDO expands human CD4+CD25high regulatory T cells by promoting maturation of LPS-treated dendritic cells. *European journal of immunology,* 37**,** 3054-62.

HIMMEL, M. E., MACDONALD, K. G., GARCIA, R. V., STEINER, T. S. & LEVINGS, M. K. 2013. Helios+ and Helios- cells coexist within the natural FOXP3+ T regulatory cell subset in humans. *Journal of immunology,* 190**,** 2001-8.

HOMBACH, A. A., KOFLER, D., HOMBACH, A., RAPPL, G. & ABKEN, H. 2007. Effective proliferation of human regulatory T cells requires a strong costimulatory CD28 signal that cannot be substituted by IL-2. *Journal of immunology,* 179**,** 7924-31.

HOPFGARTEN, J., STENWALL, P. A., WIBERG, A., ANAGANDULA, M., INGVAST, S., ROSENLING, T., KORSGREN, O. & SKOG, O. 2014. Gene expression analysis of human islets in a subject at onset of type 1 diabetes. *Acta Diabetol,* 51**,** 199-204.

HOSSAIN, D. M., PANDA, A. K., MANNA, A., MOHANTY, S., BHATTACHARJEE, P., BHATTACHARYYA, S., SAHA, T., CHAKRABORTY, S., KAR, R. K., DAS, T., CHATTERJEE, S.

& SA, G. 2013. FoxP3 acts as a cotranscription factor with STAT3 in tumor-induced regulatory T cells. *Immunity,* 39**,** 1057-69.

HU, P., HAN, Z., COUVILLON, A. D., KAUFMAN, R. J. & EXTON, J. H. 2006. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. *Molecular and cellular biology,* 26**,** 3071-84.

HUANG, S. P., CHEN, J. C., WU, C. C., CHEN, C. T., TANG, N. Y., HO, Y. T., LO, C., LIN, J. P., CHUNG, J. G. & LIN, J. G. 2009. Capsaicin-induced apoptosis in human hepatoma HepG2 cells. *Anticancer Res,* 29**,** 165-74.

HUANG, Z., YANG, B., SHI, Y., CAI, B., LI, Y., FENG, W., FU, Y., LUO, L. & WANG, L. 2012. Anti-TNF-alpha therapy improves Treg and suppresses Teff in patients with rheumatoid arthritis. *Cell Immunol,* 279**,** 25-9.

HUSS, D. J., WINGER, R. C., COX, G. M., GUERAU-DE-ARELLANO, M., YANG, Y., RACKE, M. K. & LOVETT-RACKE, A. E. 2011. TGF-beta signaling via Smad4 drives IL-10 production in effector Th1 cells and reduces T-cell trafficking in EAE. *European journal of immunology,* 41**,** 2987-96.

INGLIS, J. J., SIMELYTE, E., MCCANN, F. E., CRIADO, G. & WILLIAMS, R. O. 2008. Protocol for the induction of arthritis in C57BL/6 mice. *Nat Protoc,* 3**,** 612-8.

ITO, T., HANABUCHI, S., WANG, Y. H., PARK, W. R., ARIMA, K., BOVER, L., QIN, F. X., GILLIET, M. & LIU, Y. J. 2008. Two functional subsets of FOXP3+ regulatory T cells in human thymus and periphery. *Immunity,* 28**,** 870-80.

JARNICKI, A. G., LYSAGHT, J., TODRYK, S. & MILLS, K. H. 2006. Suppression of antitumor immunity by IL-10 and TGF-beta-producing T cells infiltrating the growing tumor: influence of tumor environment on the induction of CD4+ and CD8+ regulatory T cells. *Journal of immunology,* 177**,** 896-904.

JINDADAMRONGWECH, S., THEPPARIT, C. & SMITH, D. R. 2004. Identification of GRP 78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. *Arch Virol,* 149**,** 915-27.

JONULEIT, H., SCHMITT, E., STASSEN, M., TUETTENBERG, A., KNOP, J. & ENK, A. H. 2001. Identification and functional characterization of human CD4(+)CD25(+) T cells with regulatory properties isolated from peripheral blood. *The Journal of experimental medicine,* 193**,** 1285-94.

KAMPINGA, H. H., HAGEMAN, J., VOS, M. J., KUBOTA, H., TANGUAY, R. M., BRUFORD, E. A., CHEETHAM, M. E., CHEN, B. & HIGHTOWER, L. E. 2009. Guidelines for the nomenclature of the human heat shock proteins. *Cell stress & chaperones,* 14**,** 105-11.

KANG, S. W., KIM, S. H., LEE, N., LEE, W.-W., HWANG, K.-A., SHIN, M. S., LEE, S.-H., KIM, W.-U. & KANG, I. 2012. 1,25(OH)(2) vitamin D(3) promotes FOXP3 expression via binding to vitamin D response elements in its conserved non-coding sequence region. *Journal of Immunology (Baltimore, Md. : 1950),* 188**,** 5276-5282.

KANNEGANTI, T. D. & DIXIT, V. D. 2012. Immunological complications of obesity. *Nature immunology,* 13**,** 707-12.

KENNDEY, P. A. K., D.; ZHANG, T.; AKSOY, M.O.; KELSEN, S.G. & KILPATRICK, L.E. 2013. Macrophage Cytokine And Upr Responses To Er Stress Is Dependent On M1/M2 Polarization. *Am J Res Crit Care Med,* 187.

KERN, J., UNTERGASSER, G., ZENZMAIER, C., SARG, B., GASTL, G., GUNSILIUS, E. & STEURER, M. 2009. GRP-78 secreted by tumor cells blocks the antiangiogenic activity of bortezomib. *Blood,* 114**,** 3960-7.

KHATTRI, R., AUGER, J. A., GRIFFIN, M. D., SHARPE, A. H. & BLUESTONE, J. A. 1999. Lymphoproliferative disorder in CTLA-4 knockout mice is characterized by CD28-regulated activation of Th2 responses. *Journal of immunology,* 162**,** 5784-91.

KHAW, K. T. & WAREHAM, N. 2006. Glycated hemoglobin as a marker of cardiovascular risk. *Curr Opin Lipidol,* 17**,** 637-43.

KLINGENBERG, R., LEBENS, M., HERMANSSON, A., FREDRIKSON, G. N., STRODTHOFF, D., RUDLING, M., KETELHUTH, D. F., GERDES, N., HOLMGREN, J., NILSSON, J. & HANSSON, G. K. 2010. Intranasal immunization with an apolipoprotein B-100 fusion protein induces antigen-specific regulatory T cells and reduces atherosclerosis. *Arterioscler Thromb Vasc Biol,* 30**,** 946-52.

KOMURA, T., SAKAI, Y., HONDA, M., TAKAMURA, T., WADA, T. & KANEKO, S. 2013. ER stress induced impaired TLR signaling and macrophage differentiation of human monocytes. *Cell Immunol,* 282**,** 44-52.

KUHN, R., LOHLER, J., RENNICK, D., RAJEWSKY, K. & MULLER, W. 1993. Interleukin-10-deficient mice develop chronic enterocolitis. *Cell,* 75**,** 263-74.

KYBURZ, D., CORR, M., BRINSON, D. C., VON DAMM, A., TIGHE, H. & CARSON, D. A. 1999. Human rheumatoid factor production is dependent on CD40 signaling and autoantigen. *Journal of immunology,* 163**,** 3116-22.

KYRIAKAKIS, E., CAVALLARI, M., ANDERT, J., PHILIPPOVA, M., KOELLA, C., BOCHKOV, V., ERNE, P., WILSON, S. B., MORI, L., BIEDERMANN, B. C., RESINK, T. J. & DE LIBERO, G. 2010. Invariant natural killer T cells: linking inflammation and neovascularization in human atherosclerosis. *European journal of immunology,* 40**,** 3268-79.

LADANYI, M. & PAO, W. 2008. Lung adenocarcinoma: guiding EGFR-targeted therapy and beyond. *Mod Pathol,* 21 Suppl 2**,** S16-22.

LAKOSKI, S. G., LIU, Y., BROSNIHAN, K. B. & HERRINGTON, D. M. 2008. Interleukin-10 concentration and coronary heart disease (CHD) event risk in the estrogen replacement and atherosclerosis (ERA) study. *Atherosclerosis,* 197**,** 443-7.

LAN, Q., FAN, H., QUESNIAUX, V., RYFFEL, B., LIU, Z. & ZHENG, S. G. 2012. Induced Foxp3(+) regulatory T cells: a potential new weapon to treat autoimmune and inflammatory diseases? *J Mol Cell Biol,* 4**,** 22-8.

LEE, A. S. 2014. Glucose-regulated proteins in cancer: molecular mechanisms and therapeutic potential. *Nat Rev Cancer,* 14**,** 263-76.

LEE, Y., LIM, S., HONG, E. S., KIM, J. H., MOON, M. K., CHUN, E. J., CHOI, S. I., KIM, Y. B., PARK, Y. J., PARK, K. S., JANG, H. C. & CHOI, S. H. 2014. Serum FGF21 concentration is associated with hypertriglyceridaemia, hyperinsulinaemia and pericardial fat accumulation, independently of obesity, but not with current coronary artery status. *Clin Endocrinol (Oxf),* 80**,** 57-64.

LETTERIO, J. J. & ROBERTS, A. B. 1998. Regulation of immune responses by TGF-beta. *Annual review of immunology,* 16**,** 137-61.

LEVINGS, M. K., GREGORI, S., TRESOLDI, E., CAZZANIGA, S., BONINI, C. & RONCAROLO, M. G. 2005. Differentiation of Tr1 cells by immature dendritic cells requires IL-10 but not CD25+CD4+ Tr cells. *Blood,* 105**,** 1162-9.

LEVINGS, M. K., SANGREGORIO, R., GALBIATI, F., SQUADRONE, S., DE WAAL MALEFYT, R. & RONCAROLO, M.-G. 2001. IFN-α and IL-10 Induce the Differentiation of Human Type 1 T Regulatory Cells. *The Journal of Immunology,* 166**,** 5530-5539.

LEVITZ, S. M., TABUNI, A., NONG, S. H. & GOLENBOCK, D. T. 1996. Effects of interleukin-10 on human peripheral blood mononuclear cell responses to Cryptococcus

neoformans, Candida albicans, and lipopolysaccharide. *Infection and immunity,* 64**,** 945-51.

LEWKOWICZ, P., LEWKOWICZ, N., SASIAK, A. & TCHORZEWSKI, H. 2006. Lipopolysaccharide-activated CD4+CD25+ T regulatory cells inhibit neutrophil function and promote their apoptosis and death. *Journal of immunology,* 177**,** 7155-63.

LIN, J., LI, M., WANG, Z., HE, S., MA, X. & LI, D. 2010. The role of CD4+CD25+ regulatory T cells in macrophage-derived foam-cell formation. *J Lipid Res,* 51**,** 1208-17.

LIU, G., MA, H., QIU, L., LI, L., CAO, Y., MA, J. & ZHAO, Y. 2011. Phenotypic and functional switch of macrophages induced by regulatory CD4+CD25+ T cells in mice. *Immunol Cell Biol,* 89**,** 130-42.

LIU, W., PUTNAM, A. L., XU-YU, Z., SZOT, G. L., LEE, M. R., ZHU, S., GOTTLIEB, P. A., KAPRANOV, P., GINGERAS, T. R., FAZEKAS DE ST GROTH, B., CLAYBERGER, C., SOPER, D. M., ZIEGLER, S. F. & BLUESTONE, J. A. 2006. CD127 expression inversely correlates with FoxP3 and suppressive function of human CD4+ T reg cells. *The Journal of experimental medicine,* 203**,** 1701-11.

LONG, S. A., RIECK, M., TATUM, M., BOLLYKY, P. L., WU, R. P., MULLER, I., HO, J. C., SHILLING, H. G. & BUCKNER, J. H. 2011. Low-dose antigen promotes induction of FOXP3 in human CD4+ T cells. *Journal of immunology,* 187**,** 3511-20.

LU, M. C., LAI, N. S., YU, H. C., HUANG, H. B., HSIEH, S. C. & YU, C. L. 2010. Anti-citrullinated protein antibodies bind surface-expressed citrullinated Grp78 on monocyte/macrophages and stimulate tumor necrosis factor alpha production. *Arthritis and rheumatism,* 62**,** 1213-23.

LUMENG, C. N., BODZIN, J. L. & SALTIEL, A. R. 2007. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *Journal of Clinical Investigation,* 117**,** 175-84.

MAGG, T., MANNERT, J., ELLWART, J. W., SCHMID, I. & ALBERT, M. H. 2012. Subcellular localization of FOXP3 in human regulatory and nonregulatory T cells. *European journal of immunology,* 42**,** 1627-38.

MAGISTRELLI, G., JEANNIN, P., HERBAULT, N., BENOIT DE COIGNAC, A., GAUCHAT, J. F., BONNEFOY, J. Y. & DELNESTE, Y. 1999. A soluble form of CTLA-4 generated by alternative splicing is expressed by nonstimulated human T cells. *European journal of immunology,* 29**,** 3596-602.

MAHABADI, A. A., MASSARO, J. M., ROSITO, G. A., LEVY, D., MURABITO, J. M., WOLF, P. A., O'DONNELL, C. J., FOX, C. S. & HOFFMANN, U. 2009. Association of pericardial fat, intrathoracic fat, and visceral abdominal fat with cardiovascular disease burden: the Framingham Heart Study. *European heart journal,* 30**,** 850-6.

MALAVIGE, G. N., JEEWANDARA, C., ALLES, K. M., SALIMI, M., GOMES, L., KAMALADASA, A., JAYARATNE, S. D. & OGG, G. S. 2013. Suppression of virus specific immune responses by IL-10 in acute dengue infection. *PLoS Negl Trop Dis,* 7**,** e2409.

MALHOTRA, J. D. & KAUFMAN, R. J. 2007. The endoplasmic reticulum and the unfolded protein response. *Semin Cell Dev Biol,* 18**,** 716-31.

MARCINOWSKI, M., HOLLER, M., FEIGE, M. J., BAEREND, D., LAMB, D. C. & BUCHNER, J. 2011. Substrate discrimination of the chaperone BiP by autonomous and cochaperone-regulated conformational transitions. *Nat Struct Mol Biol,* 18**,** 150-8.

MARIN-BRIGGILER, C. I., GONZALEZ-ECHEVERRIA, M. F., MUNUCE, M. J., GHERSEVICH, S., CAILLE, A. M., HELLMAN, U., CORRIGALL, V. M. & VAZQUEZ-LEVIN, M. H. 2010. Glucose-regulated protein 78 (Grp78/BiP) is secreted by human oviduct epithelial cells and the recombinant protein modulates sperm-zona pellucida binding. *Fertility and sterility,* 93**,** 1574-84.

MARTINON, F. & GLIMCHER, L. H. 2011. Regulation of innate immunity by signaling pathways emerging from the endoplasmic reticulum. *Current opinion in immunology,* 23**,** 35-40.

MATARESE, G., CARRIERI, P. B., LA CAVA, A., PERNA, F., SANNA, V., DE ROSA, V., AUFIERO, D., FONTANA, S. & ZAPPACOSTA, S. 2005. Leptin increase in multiple sclerosis associates with reduced number of CD4+CD25+ regulatory T cells. *Proceedings of the National Academy of Sciences of the United States of America,* 102**,** 5150-5155.

MATSUMOTO, A. & HANAWALT, P. C. 2000. Histone H3 and heat shock protein GRP78 are selectively cross-linked to DNA by photoactivated gilvocarcin V in human fibroblasts. *Cancer Res,* 60**,** 3921-6.

MAZUREK, T., ZHANG, L., ZALEWSKI, A., MANNION, J. D., DIEHL, J. T., ARAFAT, H., SAROV-BLAT, L., O'BRIEN, S., KEIPER, E. A., JOHNSON, A. G., MARTIN, J., GOLDSTEIN, B. J. & SHI, Y. 2003. Human epicardial adipose tissue is a source of inflammatory mediators. *Circulation,* 108**,** 2460-6.

MCGOVERN, J. L., NGUYEN, D. X., NOTLEY, C. A., MAURI, C., ISENBERG, D. A. & EHRENSTEIN, M. R. 2012. Th17 cells are restrained by Treg cells via the inhibition of interleukin-6 in patients with rheumatoid arthritis responding to anti-tumor necrosis factor antibody therapy. *Arthritis and rheumatism,* 64**,** 3129-38.

MCMURCHY, A. N., GILLIES, J., GIZZI, M. C., RIBA, M., GARCIA-MANTEIGA, J. M., CITTARO, D., LAZAREVIC, D., DI NUNZIO, S., PIRAS, I. S., BULFONE, A., RONCAROLO, M. G., STUPKA, E., BACCHETTA, R. & LEVINGS, M. K. 2013. A novel function for FOXP3 in humans: intrinsic regulation of conventional T cells. *Blood,* 121**,** 1265-75.

MCMURCHY, A. N. & LEVINGS, M. K. 2012. Suppression assays with human T regulatory cells: a technical guide. *European journal of immunology,* 42**,** 27-34.

MENU, P., MAYOR, A., ZHOU, R., TARDIVEL, A., ICHIJO, H., MORI, K. & TSCHOPP, J. 2012. ER stress activates the NLRP3 inflammasome via an UPR-independent pathway. *Cell Death Dis,* 3**,** e261.

MEZRICH, J. D., FECHNER, J. H., ZHANG, X., JOHNSON, B. P., BURLINGHAM, W. J. & BRADFIELD, C. A. 2010. An interaction between kynurenine and the aryl hydrocarbon receptor can generate regulatory T cells. *Journal of immunology,* 185**,** 3190-8.

MIAO, C., CHEN, S., DING, J., LIU, K., LI, D., MACEDO, R., LAI, S., VOGEL-CLAUSSEN, J., BROWN, E. R., LIMA, J. A. & BLUEMKE, D. A. 2011. The association of pericardial fat with coronary artery plaque index at MR imaging: The Multi-Ethnic Study of Atherosclerosis (MESA). *Radiology,* 261**,** 109-15.

MILPIED, P., RENAND, A., BRUNEAU, J., MENDES-DA-CRUZ, D. A., JACQUELIN, S., ASNAFI, V., RUBIO, M. T., MACINTYRE, E., LEPELLETIER, Y. & HERMINE, O. 2009. Neuropilin-1 is not a marker of human Foxp3+ Treg. *European journal of immunology,* 39**,** 1466-71.

MOR, A., PLANER, D., LUBOSHITS, G., AFEK, A., METZGER, S., CHAJEK-SHAUL, T., KEREN, G. & GEORGE, J. 2007. Role of naturally occurring CD4+ CD25+ regulatory T cells in experimental atherosclerosis. *Arterioscler Thromb Vasc Biol,* 27**,** 893-900.

MOSMANN, T. R. & FONG, T. A. 1989. Specific assays for cytokine production by T cells. *J Immunol Methods,* 116**,** 151-8.

MUNRO, S. & PELHAM, H. R. 1986. An Hsp70-like protein in the ER: identity with the 78 kd glucose-regulated protein and immunoglobulin heavy chain binding protein. *Cell,* 46**,** 291-300.

MUNRO, S. & PELHAM, H. R. 1987. A C-terminal signal prevents secretion of luminal ER proteins. *Cell,* 48**,** 899-907.

MUSSO, T., ESPINOZA-DELGADO, I., PULKKI, K., GUSELLA, G. L., LONGO, D. L. & VARESIO, L. 1990. Transforming growth factor beta downregulates interleukin-1 (IL-1)-induced IL-6 production by human monocytes. *Blood,* 76**,** 2466-9.

NADKARNI, S., MAURI, C. & EHRENSTEIN, M. R. 2007. Anti-TNF-alpha therapy induces a distinct regulatory T cell population in patients with rheumatoid arthritis via TGF-beta. *The Journal of experimental medicine,* 204**,** 33-9.

NAIDOO, N., FERBER, M., MASTER, M., ZHU, Y. & PACK, A. I. 2008. Aging impairs the unfolded protein response to sleep deprivation and leads to proapoptotic signaling. *J Neurosci,* 28**,** 6539-48.

NAKAMURA, N., CHIN, H., MIYASAKA, N. & MIURA, O. 1996. An epidermal growth factor receptor/Jak2 tyrosine kinase domain chimera induces tyrosine phosphorylation of Stat5 and transduces a growth signal in hematopoietic cells. *The Journal of biological chemistry,* 271**,** 19483-8.

NG, T. H., BRITTON, G. J., HILL, E. V., VERHAGEN, J., BURTON, B. R. & WRAITH, D. C. 2013. Regulation of adaptive immunity; the role of interleukin-10. *Front Immunol,* 4**,** 129.

NI, M., ZHANG, Y. & LEE, A. S. 2011. Beyond the endoplasmic reticulum: atypical GRP78 in cell viability, signalling and therapeutic targeting. *Biochem J,* 434**,** 181-8.

NIE, H., ZHENG, Y., LI, R., GUO, T. B., HE, D., FANG, L., LIU, X., XIAO, L., CHEN, X., WAN, B., CHIN, Y. E. & ZHANG, J. Z. 2013. Phosphorylation of FOXP3 controls regulatory T cell function and is inhibited by TNF-alpha in rheumatoid arthritis. *Nature medicine,* 19**,** 322-8.

OBERLE, N., EBERHARDT, N., FALK, C. S., KRAMMER, P. H. & SURI-PAYER, E. 2007. Rapid suppression of cytokine transcription in human CD4+CD25 T cells by CD4+Foxp3+ regulatory T cells: independence of IL-2 consumption, TGF-beta, and various inhibitors of TCR signaling. *Journal of immunology,* 179**,** 3578-87.

OYADOMARI, S., TAKEDA, K., TAKIGUCHI, M., GOTOH, T., MATSUMOTO, M., WADA, I., AKIRA, S., ARAKI, E. & MORI, M. 2001. Nitric oxide-induced apoptosis in pancreatic beta cells is mediated by the endoplasmic reticulum stress pathway. *Proceedings of the National Academy of Sciences of the United States of America,* 98**,** 10845-50.

PALLOTTA, M. T., ORABONA, C., VOLPI, C., GROHMANN, U., PUCCETTI, P. & FALLARINO, F. 2010. Proteasomal Degradation of Indoleamine 2,3-Dioxygenase in CD8 Dendritic Cells is Mediated by Suppressor of Cytokine Signaling 3 (SOCS3). *Int J Tryptophan Res,* 3**,** 91-7.

PALLOTTA, M. T., ORABONA, C., VOLPI, C., VACCA, C., BELLADONNA, M. L., BIANCHI, R., SERVILLO, G., BRUNACCI, C., CALVITTI, M., BICCIATO, S., MAZZA, E. M., BOON, L., GRASSI, F., FIORETTI, M. C., FALLARINO, F., PUCCETTI, P. & GROHMANN, U. 2011. Indoleamine 2,3-dioxygenase is a signaling protein in long-term tolerance by dendritic cells. *Nature immunology,* 12**,** 870-8.

PARMAR, V. & SCHRÖDER, M. 2012. Sensing Endoplasmic Reticulum Stress. *In:* LÓPEZ-LARREA, C. (ed.) *Self and Nonself.* Springer US.

PENG, Y. & LI, Z. 2013. GRP78 secreted by tumor cells stimulates differentiation of bone marrow mesenchymal stem cells to cancer-associated fibroblasts. *Biochemical and biophysical research communications,* 440**,** 558-63.

PESCE, B., SOTO, L., SABUGO, F., WURMANN, P., CUCHACOVICH, M., LOPEZ, M. N., SOTELO, P. H., MOLINA, M. C., AGUILLON, J. C. & CATALAN, D. 2013. Effect of interleukin-6 receptor blockade on the balance between regulatory T cells and T helper type 17 cells in rheumatoid arthritis patients. *Clinical and experimental immunology,* 171**,** 237-42.

POUYSSEGUR, J., SHIU, R. P. & PASTAN, I. 1977. Induction of two transformation-sensitive membrane polypeptides in normal fibroblasts by a block in glycoprotein synthesis or glucose deprivation. *Cell,* 11**,** 941-7.

POWRIE, F., CARLINO, J., LEACH, M. W., MAUZE, S. & COFFMAN, R. L. 1996. A critical role for transforming growth factor-beta but not interleukin 4 in the suppression of T helper type 1-mediated colitis by CD45RB(low) CD4+ T cells. *The Journal of experimental medicine,* 183**,** 2669-74.

REGATEIRO, F. S., HOWIE, D., NOLAN, K. F., AGOROGIANNIS, E. I., GREAVES, D. R., COBBOLD, S. P. & WALDMANN, H. 2011. Generation of anti-inflammatory adenosine by leukocytes is regulated by TGF-beta. *European journal of immunology,* 41**,** 2955-65.

RITOSSA, F. 1962. A new puffing pattern induced by temperature shock and DNP in drosophila. *Experientia,* 18**,** 571-573.

RON, D. & HUBBARD, S. R. 2008. How IRE1 reacts to ER stress. *Cell,* 132**,** 24-6.

ROSITO, G. A., MASSARO, J. M., HOFFMANN, U., RUBERG, F. L., MAHABADI, A. A., VASAN, R. S., O'DONNELL, C. J. & FOX, C. S. 2008. Pericardial fat, visceral abdominal fat, cardiovascular disease risk factors, and vascular calcification in a community-based sample: the Framingham Heart Study. *Circulation,* 117**,** 605-13.

SALIBA, W., BARNETT-GRINESS, O. & RENNERT, G. 2013. The association between obesity and urinary tract infection. *Eur J Intern Med,* 24**,** 127-31.

SAMSON, M., AUDIA, S., JANIKASHVILI, N., CIUDAD, M., TRAD, M., FRASZCZAK, J., ORNETTI, P., MAILLEFERT, J. F., MIOSSEC, P. & BONNOTTE, B. 2012. Brief report: inhibition of interleukin-6 function corrects Th17/Treg cell imbalance in patients with rheumatoid arthritis. *Arthritis and rheumatism,* 64**,** 2499-503.

SARAIVA, M. & O'GARRA, A. 2010. The regulation of IL-10 production by immune cells. *Nature reviews. Immunology,* 10**,** 170-181.

SAVAGE, N. D., DE BOER, T., WALBURG, K. V., JOOSTEN, S. A., VAN MEIJGAARDEN, K., GELUK, A. & OTTENHOFF, T. H. 2008. Human anti-inflammatory macrophages induce Foxp3+ GITR+ CD25+ regulatory T cells, which suppress via membrane-bound TGFbeta-1. *Journal of immunology,* 181**,** 2220-6.

SCHONTHAL, A. H. 2012. Endoplasmic reticulum stress: its role in disease and novel prospects for therapy. *Scientifica (Cairo),* 2012**,** 857516.

SCHRODER, M. & KAUFMAN, R. J. 2005. The mammalian unfolded protein response. *Annu Rev Biochem,* 74**,** 739-89.

SEDDIKI, N., SANTNER-NANAN, B., MARTINSON, J., ZAUNDERS, J., SASSON, S., LANDAY, A., SOLOMON, M., SELBY, W., ALEXANDER, S. I., NANAN, R., KELLEHER, A. & FAZEKAS DE ST GROTH, B. 2006. Expression of interleukin (IL)-2 and IL-7 receptors discriminates between human regulatory and activated T cells. *The Journal of experimental medicine,* 203**,** 1693-700.

SHARMA, M. D., HOU, D. Y., LIU, Y., KONI, P. A., METZ, R., CHANDLER, P., MELLOR, A. L., HE, Y. & MUNN, D. H. 2009. Indoleamine 2,3-dioxygenase controls conversion of Foxp3+ Tregs to TH17-like cells in tumor-draining lymph nodes. *Blood,* 113**,** 6102-11.

SHIELDS, A. M., KLAVINSKIS, L. S., ANTONIOU, M., WOOLEY, P. H., COLLINS, H. L., PANAYI, G. S., THOMPSON, S. J. & CORRIGALL, V. M. 2015. Systemic gene transfer of binding immunoglobulin protein (BiP) prevents disease progression in murine collagen-induced arthritis. *Clinical and experimental immunology,* 179**,** 210-9.

SHIELDS, A. M., THOMPSON, S. J., PANAYI, G. S. & CORRIGALL, V. M. 2011. Pro-resolution immunological networks: binding immunoglobulin protein and other resolution-associated molecular patterns. *Rheumatology*.

SHIELDS, A. M., THOMPSON, S. J., PANAYI, G. S. & CORRIGALL, V. M. 2012. Pro-resolution immunological networks: binding immunoglobulin protein and other resolution-associated molecular patterns. *Rheumatology,* 51**,** 780-8.

SHIN, B. K., WANG, H., YIM, A. M., LE NAOUR, F., BRICHORY, F., JANG, J. H., ZHAO, R., PURAVS, E., TRA, J., MICHAEL, C. W., MISEK, D. E. & HANASH, S. M. 2003. Global profiling of the cell surface proteome of cancer cells uncovers an abundance of proteins with chaperone function. *The Journal of biological chemistry,* 278**,** 7607-16.

SHODA, H., FUJIO, K., SHIBUYA, M., OKAMURA, T., SUMITOMO, S., OKAMOTO, A., SAWADA, T. & YAMAMOTO, K. 2011. Detection of autoantibodies to citrullinated BiP in rheumatoid arthritis patients and pro-inflammatory role of citrullinated BiP in collagen-induced arthritis. *Arthritis research & therapy,* 13**,** R191.

SICARI, R., SIRONI, A. M., PETZ, R., FRASSI, F., CHUBUCHNY, V., DE MARCHI, D., POSITANO, V., LOMBARDI, M., PICANO, E. & GASTALDELLI, A. 2011. Pericardial rather than epicardial fat is a cardiometabolic risk marker: an MRI vs echo study. *J Am Soc Echocardiogr,* 24**,** 1156-62.

STERNBERG, N. 1973. Properties of a mutant of Escherichia coli defective in bacteriophage lambda head formation (groE). II. The propagation of phage lambda. *J Mol Biol,* 76**,** 25-44.

SUN, F. C., WEI, S., LI, C. W., CHANG, Y. S., CHAO, C. C. & LAI, Y. K. 2006. Localization of GRP78 to mitochondria under the unfolded protein response. *Biochem J,* 396**,** 31-9.

SZANTO, S., KORENY, T., MIKECZ, K., GLANT, T. T., SZEKANECZ, Z. & VARGA, J. 2007. Inhibition of indoleamine 2,3-dioxygenase-mediated tryptophan catabolism accelerates collagen-induced arthritis in mice. *Arthritis research & therapy,* 9**,** R50.

TAKEMOTO, H., YOSHIMORI, T., YAMAMOTO, A., MIYATA, Y., YAHARA, I., INOUE, K. & TASHIRO, Y. 1992. Heavy chain binding protein (BiP/GRP78) and endoplasmin are exported from the endoplasmic reticulum in rat exocrine pancreatic cells, similar to protein disulfide-isomerase. *Arch Biochem Biophys,* 296**,** 129-36.

TERNESS, P., BAUER, T. M., ROSE, L., DUFTER, C., WATZLIK, A., SIMON, H. & OPELZ, G. 2002. Inhibition of allogeneic T cell proliferation by indoleamine 2,3-dioxygenase-

expressing dendritic cells: mediation of suppression by tryptophan metabolites. *The Journal of experimental medicine,* 196**,** 447-57.

THIOLAT, A., SEMERANO, L., PERS, Y. M., BITON, J., LEMEITER, D., PORTALES, P., QUENTIN, J., JORGENSEN, C., DECKER, P., BOISSIER, M. C., LOUIS-PLENCE, P. & BESSIS, N. 2014. Interleukin-6 receptor blockade enhances CD39+ regulatory T cell development in rheumatoid arthritis and in experimental arthritis. *Arthritis Rheumatol,* 66**,** 273-83.

THORNTON, A. M., KORTY, P. E., TRAN, D. Q., WOHLFERT, E. A., MURRAY, P. E., BELKAID, Y. & SHEVACH, E. M. 2010. Expression of Helios, an Ikaros transcription factor family member, differentiates thymic-derived from peripherally induced Foxp3+ T regulatory cells. *Journal of immunology,* 184**,** 3433-41.

TING, J. & LEE, A. S. 1988. Human gene encoding the 78,000-dalton glucose-regulated protein and its pseudogene: structure, conservation, and regulation. *DNA,* 7**,** 275-86.

TODD, D. J., KNOWLTON, N., AMATO, M., FRANK, M. B., SCHUR, P. H., IZMAILOVA, E. S., ROUBENOFF, R., SHADICK, N. A., WEINBLATT, M. E., CENTOLA, M. & LEE, D. M. 2011. Erroneous augmentation of multiplex assay measurements in patients with rheumatoid arthritis due to heterophilic binding by serum rheumatoid factor. *Arthritis and rheumatism,* 63**,** 894-903.

TRAN, D. Q., RAMSEY, H. & SHEVACH, E. M. 2007. Induction of FOXP3 expression in naive human CD4+FOXP3 T cells by T-cell receptor stimulation is transforming growth factor-beta dependent but does not confer a regulatory phenotype. *Blood,* 110**,** 2983-90.

TRIANTAFILOU, K., FRADELIZI, D., WILSON, K. & TRIANTAFILOU, M. 2002. GRP78, a coreceptor for coxsackievirus A9, interacts with major histocompatibility complex class I molecules which mediate virus internalization. *J Virol,* 76**,** 633-43.

UDONO, H. 2012. Heat shock protein magic in antigen trafficking within dendritic cells: implications in antigen cross-presentation in immunity. *Acta Med Okayama,* 66**,** 1-6.

VALENCIA, X., STEPHENS, G., GOLDBACH-MANSKY, R., WILSON, M., SHEVACH, E. M. & LIPSKY, P. E. 2006. TNF downmodulates the function of human CD4+CD25hi T-regulatory cells. *Blood,* 108**,** 253-61.

VAN AMELSFORT, J. M., VAN ROON, J. A., NOORDEGRAAF, M., JACOBS, K. M., BIJLSMA, J. W., LAFEBER, F. P. & TAAMS, L. S. 2007. Proinflammatory mediator-induced reversal

of CD4+,CD25+ regulatory T cell-mediated suppression in rheumatoid arthritis. *Arthritis and rheumatism,* 56**,** 732-42.

VAN DER WEERD, K., DIK, W. A., SCHRIJVER, B., SCHWEITZER, D. H., LANGERAK, A. W., DREXHAGE, H. A., KIEWIET, R. M., VAN AKEN, M. O., VAN HUISSTEDE, A., VAN DONGEN, J. J., VAN DER LELIJ, A. J., STAAL, F. J. & VAN HAGEN, P. M. 2012. Morbidly obese human subjects have increased peripheral blood CD4+ T cells with skewing toward a Treg- and Th2-dominated phenotype. *Diabetes,* 61**,** 401-8.

VAN HERWIJNEN, M. J., WIETEN, L., VAN DER ZEE, R., VAN KOOTEN, P. J., WAGENAAR-HILBERS, J. P., HOEK, A., DEN BRABER, I., ANDERTON, S. M., SINGH, M., MEIRING, H. D., VAN ELS, C. A., VAN EDEN, W. & BROERE, F. 2012. Regulatory T cells that recognize a ubiquitous stress-inducible self-antigen are long-lived suppressors of autoimmune arthritis. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 14134-9.

VAN KERKHOVE, M. D., VANDEMAELE, K. A. H., SHINDE, V., JARAMILLO-GUTIERREZ, G., KOUKOUNARI, A., DONNELLY, C. A., CARLINO, L. O., OWEN, R., PATERSON, B., PELLETIER, L., VACHON, J., GONZALEZ, C., HONGJIE, Y., ZIJIAN, F., CHUANG, S. K., AU, A., BUDA, S., KRAUSE, G., HAAS, W., BONMARIN, I., TANIGUICHI, K., NAKAJIMA, K., SHOBAYASHI, T., TAKAYAMA, Y., SUNAGAWA, T., HERAUD, J. M., ORELLE, A., PALACIOS, E., VAN DER SANDE, M. A. B., WIELDERS, C. C. H. L., HUNT, D., CUTTER, J., LEE, V. J., THOMAS, J., SANTA-OLALLA, P., SIERRA-MOROS, M. J., HANSHAOWORAKUL, W., UNGCHUSAK, K., PEBODY, R., JAIN, S., MOUNTS, A. W. & ON BEHALF OF THE, W. H. O. W. G. F. R. F. F. S. H. N. P. I. 2011. Risk Factors for Severe Outcomes following 2009 Influenza A (H1N1) Infection: A Global Pooled Analysis. *PLoS Med,* 8**,** e1001053.

VAN VLASSELAER, P., PUNNONEN, J. & DE VRIES, J. E. 1992. Transforming growth factor-beta directs IgA switching in human B cells. *The Journal of Immunology,* 148**,** 2062-7.

VERCOULEN, Y., WEHRENS, E. J., VAN TEIJLINGEN, N. H., DE JAGER, W., BEEKMAN, J. M. & PRAKKEN, B. J. 2009. Human regulatory T cell suppressive function is independent of apoptosis induction in activated effector T cells. *PLoS One,* 4**,** e7183.

VERMA, G. & DATTA, M. 2010. IL-1beta induces ER stress in a JNK dependent manner that determines cell death in human pancreatic epithelial MIA PaCa-2 cells. *Apoptosis,* 15**,** 864-76.

VERMA, N. D., PLAIN, K. M., NOMURA, M., TRAN, G. T., ROBINSON, C., BOYD, R., HODGKINSON, S. J. & HALL, B. M. 2009. CD4+CD25+ T cells alloactivated ex vivo by IL-2 or IL-4 become potent alloantigen-specific inhibitors of rejection with different phenotypes, suggesting separate pathways of activation by Th1 and Th2 responses. *Blood,* 113**,** 479-87.

WACHSTEIN, J., TISCHER, S., FIGUEIREDO, C., LIMBOURG, A., FALK, C., IMMENSCHUH, S., BLASCZYK, R. & EIZ-VESPER, B. 2012. HSP70 Enhances Immunosuppressive Function of CD4(+)CD25(+)FoxP3(+) T Regulatory Cells and Cytotoxicity in CD4(+)CD25(-) T Cells. *PLoS One,* 7**,** e51747.

WAGNER, N. M., BRANDHORST, G., CZEPLUCH, F., LANKEIT, M., EBERLE, C., HERZBERG, S., FAUSTIN, V., RIGGERT, J., OELLERICH, M., HASENFUSS, G., KONSTANTINIDES, S. & SCHAFER, K. 2013. Circulating regulatory T cells are reduced in obesity and may identify subjects at increased metabolic and cardiovascular risk. *Obesity (Silver Spring),* 21**,** 461-8.

WALTER, G. J., EVANS, H. G., MENON, B., GULLICK, N. J., KIRKHAM, B. W., COPE, A. P., GEISSMANN, F. & TAAMS, L. S. 2013. Interaction with activated monocytes enhances cytokine expression and suppressive activity of human CD4+CD45ro+CD25+CD127(low) regulatory T cells. *Arthritis and rheumatism,* 65**,** 627-38.

WAN, Y. Y. & FLAVELL, R. A. 2007. Regulatory T-cell functions are subverted and converted owing to attenuated Foxp3 expression. *Nature,* 445**,** 766-70.

WANG, J., HUIZINGA, T. W. & TOES, R. E. 2009. De novo generation and enhanced suppression of human CD4+CD25+ regulatory T cells by retinoic acid. *Journal of immunology,* 183**,** 4119-26.

WANG, J., IOAN-FACSINAY, A., VAN DER VOORT, E. I., HUIZINGA, T. W. & TOES, R. E. 2007. Transient expression of FOXP3 in human activated nonregulatory CD4+ T cells. *European journal of immunology,* 37**,** 129-38.

WANG, P., WU, P., SIEGEL, M. I., EGAN, R. W. & BILLAH, M. M. 1995. Interleukin (IL)-10 inhibits nuclear factor kappa B (NF kappa B) activation in human monocytes. IL-10 and IL-4 suppress cytokine synthesis by different mechanisms. *The Journal of biological chemistry,* 270**,** 9558-63.

WANG, T., SUN, X., ZHAO, J., ZHANG, J., ZHU, H., LI, C., GAO, N., JIA, Y., XU, D., HUANG, F. P., LI, N., LU, L. & LI, Z. G. 2014. Regulatory T cells in rheumatoid arthritis showed increased plasticity toward Th17 but retained suppressive function in peripheral blood. *Annals of the rheumatic diseases*.

WARD, F. J., DAHAL, L. N., WIJESEKERA, S. K., ABDUL-JAWAD, S. K., KAEWARPAI, T., XU, H., VICKERS, M. A. & BARKER, R. N. 2013. The soluble isoform of CTLA-4 as a regulator of T-cell responses. *European journal of immunology*.

WEHRENS, E. J., MIJNHEER, G., DUURLAND, C. L., KLEIN, M., MEERDING, J., VAN LOOSDREGT, J., DE JAGER, W., SAWITZKI, B., COFFER, P. J., VASTERT, B., PRAKKEN, B. J. & VAN WIJK, F. 2011. Functional human regulatory T cells fail to control autoimmune inflammation due to PKB/c-akt hyperactivation in effector cells. *Blood,* 118**,** 3538-48.

WELSH, P., MURRAY, H. M., FORD, I., TROMPET, S., DE CRAEN, A. J., JUKEMA, J. W., STOTT, D. J., MCINNES, I. B., PACKARD, C. J., WESTENDORP, R. G. & SATTAR, N. 2011. Circulating interleukin-10 and risk of cardiovascular events: a prospective study in the elderly at risk. *Arterioscler Thromb Vasc Biol,* 31**,** 2338-44.

WERSTUCK, G. H., LENTZ, S. R., DAYAL, S., HOSSAIN, G. S., SOOD, S. K., SHI, Y. Y., ZHOU, J., MAEDA, N., KRISANS, S. K., MALINOW, M. R. & AUSTIN, R. C. 2001. Homocysteine-induced endoplasmic reticulum stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways. *Journal of Clinical Investigation,* 107**,** 1263-73.

WILDIN, R. S., SMYK-PEARSON, S. & FILIPOVICH, A. H. 2002. Clinical and molecular features of the immunodysregulation, polyendocrinopathy, enteropathy, X linked (IPEX) syndrome. *J Med Genet,* 39**,** 537-45.

WING, K., ONISHI, Y., PRIETO-MARTIN, P., YAMAGUCHI, T., MIYARA, M., FEHERVARI, Z., NOMURA, T. & SAKAGUCHI, S. 2008. CTLA-4 control over Foxp3+ regulatory T cell function. *Science,* 322**,** 271-5.

XIAO, G., CHUNG, T. F., PYUN, H. Y., FINE, R. E. & JOHNSON, R. J. 1999. KDEL proteins are found on the surface of NG108-15 cells. *Brain Res Mol Brain Res,* 72**,** 121-8.

XIAO, Y. Q., FREIRE-DE-LIMA, C. G., SCHIEMANN, W. P., BRATTON, D. L., VANDIVIER, R. W. & HENSON, P. M. 2008. Transcriptional and translational regulation of TGF-beta production in response to apoptotic cells. *Journal of immunology,* 181**,** 3575-85.

YADAV, M., LOUVET, C., DAVINI, D., GARDNER, J. M., MARTINEZ-LLORDELLA, M., BAILEY-BUCKTROUT, S., ANTHONY, B. A., SVERDRUP, F. M., HEAD, R., KUSTER, D. J., RUMINSKI, P., WEISS, D., VON SCHACK, D. & BLUESTONE, J. A. 2012. Neuropilin-1 distinguishes natural and inducible regulatory T cells among regulatory T cell subsets in vivo. *The Journal of experimental medicine,* 209**,** 1713-22.

YANG, S., VIGERUST, D. J. & SHEPHERD, V. L. 2013. Interaction of members of the heat shock protein-70 family with the macrophage mannose receptor. *Journal of leukocyte biology,* 93**,** 529-36.

YEHUDA-SHNAIDMAN, E. & SCHWARTZ, B. 2012. Mechanisms linking obesity, inflammation and altered metabolism to colon carcinogenesis. *Obes Rev,* 13**,** 1083-95.

YOO, S. A., YOU, S., YOON, H. J., KIM, D. H., KIM, H. S., LEE, K., AHN, J. H., HWANG, D., LEE, A. S., KIM, K. J., PARK, Y. J., CHO, C. S. & KIM, W. U. 2012. A novel pathogenic role of the ER chaperone GRP78/BiP in rheumatoid arthritis. *The Journal of experimental medicine*.

YOSHIDA, K., OCHIAI, A., MATSUNO, H., PANAYI, G. S. & CORRIGALL, V. M. 2011. Binding immunoglobulin protein resolves rheumatoid synovitis: a xenogeneic study using rheumatoid arthritis synovial membrane transplants in SCID mice. *Arthritis research & therapy,* 13**,** R149.

YU, N., LI, X., SONG, W., LI, D., YU, D., ZENG, X., LI, M. & LENG, X. 2012. CD4(+)CD25 (+)CD127 (low/-) T cells: a more specific Treg population in human peripheral blood. *Inflammation,* 35**,** 1773-80.

YUAN, X. & MALEK, T. R. 2012. Cellular and molecular determinants for the development of natural and induced regulatory T cells. *Human immunology,* 73**,** 773-82.

YUN, C. H., LIN, T. Y., WU, Y. J., LIU, C. C., KUO, J. Y., YEH, H. I., YANG, F. S., CHEN, S. C., HOU, C. J., BEZERRA, H. G., HUNG, C. L. & CURY, R. C. 2012. Pericardial and thoracic peri-aortic adipose tissues contribute to systemic inflammation and calcified coronary atherosclerosis independent of body fat composition, anthropometric measures and traditional cardiovascular risks. *Eur J Radiol,* 81**,** 749-56.

ZAISS, D. M., VAN LOOSDREGT, J., GORLANI, A., BEKKER, C. P., GRONE, A., SIBILIA, M., VAN BERGEN EN HENEGOUWEN, P. M., ROOVERS, R. C., COFFER, P. J. & SIJTS, A. J. 2013. Amphiregulin enhances regulatory T cell-suppressive function via the epidermal growth factor receptor. *Immunity,* 38**,** 275-84.

ZANIN-ZHOROV, A., CAHALON, L., TAL, G., MARGALIT, R., LIDER, O. & COHEN, I. R. 2006. Heat shock protein 60 enhances CD4+ CD25+ regulatory T cell function via innate TLR2 signaling. *Journal of Clinical Investigation,* 116**,** 2022-32.

ZANIN-ZHOROV, A., DING, Y., KUMARI, S., ATTUR, M., HIPPEN, K. L., BROWN, M., BLAZAR, B. R., ABRAMSON, S. B., LAFAILLE, J. J. & DUSTIN, M. L. 2010. Protein kinase C-theta mediates negative feedback on regulatory T cell function. *Science,* 328**,** 372-6.

ZANIN-ZHOROV, A., LIN, J., SCHER, J., KUMARI, S., BLAIR, D., HIPPEN, K. L., BLAZAR, B. R., ABRAMSON, S. B., LAFAILLE, J. J. & DUSTIN, M. L. 2012. Scaffold protein Disc large homolog 1 is required for T-cell receptor-induced activation of regulatory T-cell function. *Proceedings of the National Academy of Sciences of the United States of America,* 109**,** 1625-30.

ZHANG, Y., CHO, Y. Y., PETERSEN, B. L., ZHU, F. & DONG, Z. 2004. Evidence of STAT1 phosphorylation modulated by MAPKs, MEK1 and MSK1. *Carcinogenesis,* 25**,** 1165-75.

ZHENG, S. G., GRAY, J. D., OHTSUKA, K., YAMAGIWA, S. & HORWITZ, D. A. 2002. Generation ex vivo of TGF-beta-producing regulatory T cells from CD4+CD25- precursors. *Journal of immunology,* 169**,** 4183-9.

ZHENG, S. G., WANG, J. H., GRAY, J. D., SOUCIER, H. & HORWITZ, D. A. 2004. Natural and Induced CD4+CD25+ Cells Educate CD4+CD25− Cells to Develop Suppressive Activity: The Role of IL-2, TGF-β, and IL-10. *The Journal of Immunology,* 172**,** 5213-5221.

ZIMMERMANN, M. D., J. 2009. HSPA5 (heat shock 70kDa protein 5 (glucose-regulated protein, 78kDa)). *Atlas Genet Cytogenet Oncol Haematol,* 14.

Chapter 9

Appendix

9 Appendix

9.1 List of recipes and buffers

9.1.1 Cell isolation

MACS buffer - MACS buffer – Dulbecco's PBS 1X without Calcium and Magnesium (PAA, Pasching, Austria) + 2 mM of EDTA (Lonza) and 0.5% Bovine Serum Albumin (gold standard) (PAA)

9.1.2 FACS

Washing buffer - Dulbecco's PBS 1X without Calcium and Magnesium (PAA) + 0.1% sodium azide (Sigma Aldrich)

Staining buffer – Washing buffer added + 2% BSA (PAA)

Washing buffer – 1x PBS (PAA) added with 0.5% sodium azide (Acros Organics, Short Hills, USA)

9.1.3 ELISA

Washing buffer for IFN-γ, TNF-α, IL-1β and supernatant TGF-β1 – 5 mL Tween 20 (Fischer Scientific, Waltham, USA) was added into 10 L of 1x PBS

10x PBS – 80 g of sodium chloride (VWR International, Radnor, USA), 2 g of potassium chloride (VWR), 29 g of di-sodium hydrogen phosphate and 2 g of potassium dihydrogen phosphate (VWR) were added into 1 L of deionized water and pH was adjusted to 6.7

2N Sulphuric Acid (H_2SO_4) – 110 mL of concentrated sulphuric acid (Fisher Scientific, Loughborough, UK) was added into purified water up to 1L.

2N Hydrochloric Acid (HCl) – 88.8 mL of concentrated hydrochloric acid (Fisher Scientific) was added into purified water up to 1L.

2N Sodium hydroxide (NaOH) – 40 g of sodium hydroxide (BDH, Poole, England) was added into purified water up to 1L.

Washing buffer for circulating BiP – 2% BSA (PAA) in 1X PBS (from 10X PBS) + 0.05% Tween (Fischer Scientific)